A COMPREHENSIVE STUDY ON THE ROLE OF HORMONES, SEED COAT AND GENES DURING THE GERMINATION OF CANOLA (*BRASSICA NAPUS*) SEED UNDER ADVERSE ENVIRONMENTAL CONDITIONS

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ABSTRACT

Seed vigor, although not well understood, is a key critical component for yield and is in part due to a well establishment and vigorous stand of canola (*Brassica napus*) seedling under less than ideal conditions in Western Canada. My objective was to determine what constitutes vigor by studying the response of a black seed line and a yellow seed line imbibed at 8 ºC in either water, saline or osmotic solutions, abscisic acid (ABA), ABA biosynthesis inhibitor, gibberellin (GA_{4+7}), inhibitor of GA biosynthesis and a germination promoter, fusicoccin. Also tested was the effect of seed coat (testa) on seed germination rate and percent germination. Previous studies have established that seed vigor is in part hormonal controlled and genetically controlled. In our study, gene expression was investigated by using transcriptome analysis and hormonal analysis was used to quantitate the changes in hormones and their metabolites during germination.

Both the black and the yellow canola seed lines were very sensitive to increasing concentrations of saline and osmotic solutions; however, at the same osmotic potential, osmotic solutions were more inhibitory. The yellow seed line was more sensitive to these conditions than the black seed line. As expected, ABA delayed seed germination, whereas GA_{4+7} enhanced seed germination and GA_{4+7} partially overcame the inhibitory effect of ABA. The seed coat was a major factor affecting the germination rate of the yellow seed line; however, GA_{4+7} overcame the inhibitory effect of the seed coat, whereas ABA exacerbated it. Fusicoccin was more stimulatory to germination than GA_{4+7}; however, unlike GA_{4+7}, it was unable to overcome the inhibitory effect of paclobutrazol, a GA biosynthesis inhibitor. Fluridone, an ABA biosynthesis inhibitor,

was unable to overcome the inhibitory effects of a saline solution suggesting that the inhibitory effect was not due to elevated ABA levels. Ethylene, a stimulator of germination, did not appear to be involved in the germination of these two lines. Controlled deterioration at 35 ºC, 85% RH was either partially or completely overcome by exogenous GA_{4+7}. This study demonstrates that the role of hormones, salinity and seed coat on the germination of canola seed under low temperature enviromental conditions.

During germination, ABA declined while GA_4 increased. Higher ABA was found in un-germinated seeds compared to germinated seeds. GA_{4+7} was lower in seeds imbibed in the saline solution compared to seeds imbibed in water. Un-germinated seeds imbibed in ABA had lower GA_{4+7} compared to un-germinated seeds imbibed in water; however, the contents of GA_{4+7} were similar for germinated seeds imbibed in either water or ABA. Phaseic acid (PA) and dihydrophaseic acid (DPA) increased in seeds imbibed in either water, the saline solution or ABA, while they decreased in seeds imbibed in GA_{4+7}. In addition, we found that ABA inhibited GA_4 biosynthesis, whereas, GA had no effect on ABA biosynthesis, but altered the ABA catabolic pathway.

Gene expression profiles revealed that there are significant differences between un-germinated and germinated seeds. Seeds imbibed in water, GA_{4+7}, a saline solution or ABA had different gene profiles. LEA genes, hormone-related genes, hydrolase-related genes and specific seed germination-related genes were identified and their expression profiles were finely associated with seed germination performance.

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1. Introduction

The seed is a critical stage in the plant life history in that it protects the plant from unfavorable conditions as well as its role as a dispersal unit of the next generation (Bentsink and Koornneef, 2002). Seed germination begins when quiescent dry seeds uptake water and is completed when the radicle penetrates the seed coat (testa) (Bewley, 1997a; Gallardo et al., 2001; Gallardo et al., 2002). Environmental factors such as light, water, temperatures and stressful conditions can affect the germination rate and percent of seed germination (Bewley, 1997a; Bentsink and Koornneef, 2002). The environment during seed development and maturation has a profound effect on the viability and vigor of seeds. Numerous studies have focused on the regulatory roles of hormones, proteins and genes on the control of seed germination; however, there are limited data on canola (*Brassica napus*) seed germination.

Seed vigor is the major factor affecting germination, seedling emergence, stand establishment, weed competition, carbon fixation, quality and final yield especially for small seeded crops, such as canola. Environmental constraints during seed development (embryogenesis) restrict and impair development and consequently affect seed germination and seedling emergence. Since all seeds do not develop at the same time on the plant, a population of seeds is exposed to different constraints. This partially explains why subsequent time to complete germination follows a sigmoid distribution that can range from 1 day to several months.

Canola, a small seeded oil crop, currently ranks number two in total acreage and contributes 2 to 2.6 billion dollars annually to the total revenue for Canada. However, adverse environmental spring conditions often delay germination and seedling emergence

by one to two weeks. Since germination and emergence are dependent on seed rerserves, it is a race against time for seedlings to emerge and accumulate energy from photosynthesis. Cool spring soil temperatures delay germination and emergence resulting in microbial and flea beetle attack, decreased seedling vigor, delayed maturity, and decreased yield. To conserve energy and moisture, and to reduce erosion, producers are switching to conservation tillage practices which are proving to be very successful. The downside to this practice is that soil temperatures are 2 to 5 ºC cooler than tilled soils. When maturity is delayed, the plant flowers and develops seed during the hottest and driest period of the growing season. Temperatures higher than 27 ºC results in flower abortion and reduced pod and seed set, therefore reducing seed yield and oil content (Morrison 1993; Angadi et al., 2000). Kirkland and Johnson (2000) observed canola sown in the fall prior to freeze-up flowered and matured two to three weeks earlier than canola sown in spring. Yields were 38% higher and oil content was 2% higher for fall sown canola versus spring sown canola (Gusta et al., 2004). Fall sown canola seeds were larger, had higher seed weight, and were dark in colour indicating the seeds were fully mature. Due to environmental constraints, spring sown seeds are brown in colour, small, have a low seed weight and often lack vigor. A delay in maturity from slow and poor spring establishment can result in reduced yield and unmarketable green seed due to fall frosts. The Canola Council of Canada has identified low seed vigor as a major factor limiting both stand establishment and yield in western Canada.

Low soil temperatures and salinity are considered to be major factors limiting seed germination, emergence, and stand establishment of canola in western Canada. It has long been established that the fertilizer and its site of application have a dramatic effect on seed germination and stand establishment (Ukrainetz, 1974; Rostad et al., 1995). While information about the control of seed germination has greatly increased, knowledge about the response of hormones and genes for seeds germinated under abiotic stress conditions is minimal. An understanding of the regulatory roles of hormones and genes on seed germination could be used to predict germination response and may be used by plant breeders to select superior genotype.

Hypothesis

Seed germination and germination rate are a function of gene regulation which is controlled in part by environmental factors and phytohormones.

Objectives

Physiology

To assess the role of a saline solution, osmoticum, hormones and their biosynthesis inhibitors, seed coat (or testa) and controlled deterioration (CD) on the germination of canola seed at 8 ºC.

Hormone Dynamics

To analyze changes in endogenous phytohormone profiles and determine their role in canola seed germination.

Genes Associated with Germination

By genomic (microarray) analysis to identify genes which are associated with canola seed germination at low temperature in the presence of exogenous hormones, and a saline solution.

2. Literature Review

2.1 Seed Development

Seed development or formation is important in that it determines seed vigor which affects subsequent seed germination and stand establishment (Mohamed et al., 1985; Sanhewe and Ellis, 1996). Genes that affect seed size, dormancy and germination, have been reviewed by Nonogaki (2006). Seed development is divided into two phases: early embryogenesis and maturation (Braybrook et al., 2006). In the earlier embryogenesis stage, the heart shape seed embryo is formed and thereafter, the seed enters the seed maturation stage (Goldberg et al., 1994). During this stage, storage reserves are accumulated and also most seeds acquire desiccation tolerance (Kermode, 1997; Vertucci and Farrant, 1997). Seeds with desiccation tolerance are called orthodox seeds and can be dried and stored over time, while, seeds without desiccation tolerance are called recalcitrant seeds and can not be stored in a dry stage (Vertucci and Farrant, 1997).

2.1.1 Early Seed Embryogenesis

Early embryogenesis initiates from double fertilization and ends with the heart stage (Chaudhury et al., 2001). One sperm cell fuses with the egg cell and the second sperm cell fuses with the central cell, leading to the formation of a diploid zygote and a triploid endosperm respectively in the embryo sac (Goldberg et al., 1994; Ohad et al., 1996; Berger, 1999). At this stage, the basic body plan, formation of apical-basal and radial pattern is established (Jürgens et al., 1991; Laux and Jürgens, 1997). The apical – basal pattern is arranged along the axis with the alignment of shoot meristem, cotyledons, hypocotyls, roots and root meristem from top to bottom (Jürgens et al, 1991). The radial pattern is characterized by a concentric arrangement of the primary tissues: central

conductive tissue, peripheral epidermis and underneath ground tissue (Laux and Jürgens, 1997).

The body plan set up during early embryogenesis is very important in that it determines the future development of seedlings; however, the controlling mechanisms underlying this pattern formation are poorly understood. Most of the knowledge about pattern formation comes from the study of Arabidopsis mutants. Mutation in the *GNOM/EMB30 (GN)* gene affects the apical-basal polarity of embryo and mutation in the *GURKE (GK)* gene has a specific effect on the development of apical domain (Laux and Jürgens, 1997). Four genes, *WUSCHEL (WUS), ZWILLE (ZIL), Shoot Meristemless (STM*) and *AINTEGUMENTA (ANT)* are suggested to function in shoot meristem organization and defects in these genes result in an abnormal organization of the shoot meristem (Laux and Jürgens, 1997; Jürgens, 2001). Three genes *MONOPTEROS (MP), BODENLOS (BDL)* and *AUXIN RESISTANT 6 (AXR6)* are required for the proper development of the root meristem (Jürgens, 2001). Abnormal radial pattern formation is displayed by mutations in the following genes: *PINOCCHIO (PIO), SCRECROW (SCR)* and *WOODEN LEG (WOL),* indicating regulatory roles of these genes in the establishment of radial pattern (Laux and Jürgens, 1997). Studies on early embryogenesis have been greatly improved by analyzing these specific mutant phenotypes (Laux and Jürgens, 1997). However, molecular studies have yet to define or resolve how these genes control the body plan. Further molecular studies should provide an insight on the function of these genes and will also facilitate testing the genetic models of cell interactions.

2.1.2 Endosperm Development

Endosperm development is another important event during early embryogenesis. Endosperm development is classically dived into four stages: syncytical, cellularization, differentiation and death (Berger, 1999; Chaudhury et al., 2001). The main function of the endosperm is for storage accumulation in monocots as well as the control of nutrients to the embryo in dicots (Olsen, 1998; Hirner et al., 1998). However, studies on carrot seeds revealed that the endosperm controls embryogenesis (Van Hengel et al., 1998). Moreover, mutant studies in *Oryza sativa* and *Zea mays* indicated that the embryo and endosperm regulate the development of each other (Hong et al., 1996; Opsahi-Ferstad et al., 1997). Therefore, the function of the endosperm is more complicated than previously thought.

Information about the regulation of endosperm development is enhanced by the identification of the *fertilization- independent seed* (*fis*) class mutants which affect endosperm development (Chaudhury et al., 2001). The *FIS/MEDEA* (*MEA*) and the *FIS/ FIE fis* class genes display a gametophytic maternal-effect and mutants produce abortive seeds (Ohad et al., 1996; Chaudhury et al., 1997; Grossniklaus,1998, 2001; Luo et al., 1999). In Arabidopsis, the *MEDEA* (*MEA*) gene encodes a SET [*Su(var)3-9, Enhancer-of-zeste, Trithorax*] -domain, polycomb group (PcG) protein, homologous to the *Enhancer of zeste* [*E(z)*] in Drosophila (Grossniklaus et al., 1998). Mutation of the *MEA* gene results in the formation of an endosperm in the absence of fertilization and extends endosperm nucleation after fertilization (Kiyosue et al., 1999). Genetic studies characterized *MEA* as an imprinting gene that displays parent –of-origin-dependent effects specifically on the endosperm. *FIE* was identified as a gene involved in the repression of endosperm development by inhibiting transcriptional genes required for

central cell nucleus replication until fertilization occurs (Ohad et al., 1996, 1999). The product encoded by the *FIE* gene is homologous to *extra sex combs* (*esc*), WD motif – containing ploycomb proteins in Drosophila and mammals (Ohad et al., 1999). Two mechanisms were proposed to explain the effects of the *FIE* gene on repression of endosperm development. The first mechanism suggests that endosperm development is triggered by the activation of the FIE protein which is required in advance of the initiation of fertilization (Ohad et al., 1996). A mutation of *FIE* produces an active FIE which initiates endosperm development without fertilization (Ohad et al., 1996). The second mechanism suggests that fertilization inactivates the FIE protein which prevents endosperm development. Mutation of FIE abolishes the requirement of fertilization for endosperm development (Ohad et al., 1996). *FIS2*, another gene associated with prevention of endosperm development before fertilization, was characterized to encode a Zn-finger protein that acts as a transcriptional regulator (Luo et al., 1999; Grossniklaus et al., 2001). Sequence affinities between MEA and FIE and a similar function of MEA, FIE and FIS2 on endosperm development indicate these proteins may form a complex that regulates seed development (Grossniklaus et al., 2001). A yeast two-hybrid experiment revealed that MEA and FIE interact directly; however, no direct interaction of FIS2 occurs with FIE or MEA (Luo et al., 2000). A MEA and FIE complex with a 600kDa molecular weight was identified to be associated with seed development in Arabidopsis. Another protein MULTI-COPY SUPPRESSOR OF IRA1 (MSI1) is also a component of this complex (Kohler et al., 2003). Similar to *MEA*, *FIE* and *FIS2*, *MSI1* is also an imprinting gene with parent-origin-effects on seed development. Seed abortion will occur if any of the above mutant alleles is present in its mother plant (Kohler and Makarevich,

2006). This maternal effect on seed endosperm development is postulated to be controlled by DNA methylation (Adams et al., 2000; Kohler and Makarevich, 2006).

2.1.3 Seed Maturation

Following embryogenesis, cell division of the embryo is arrested and seed development enters the maturation stage (Vicente-Carbajosa and Carbonero, 2005). Seed maturation is the quiescent process in the plant life cycle which is suggested to be an evolutionary mechanism to circumvent adverse conditions until the environment is favorable to disperse the next generation (Wobus and Webber, 1999; Vicente-Carbajosa and Carbonero, 2005; Braybrook et al., 2006). Seed maturation can be divided into three phases: early, mid and late maturation (Vicente-Carbajosa and Carbonero, 2005). During early and mid maturation, storage proteins accumulate. During the late maturation stage, LEA (Late Embryogenesis Abundant) proteins, and metabolites in the form of carbohydrates and lipids, accumulate (Baud et al., 2002; Vicente-Carbajosa and Carbonero, 2005). Also, during this process, desiccation tolerance and seed dormancy are initiated (Vicente-Carbajosa and Carbonero, 2005)

Information on the regulation of these processes at the molecular levels comes from studies on Arabidopsis, grain legumes and cereals (primarily maize) (Wobus and Weber, 1999). Mutations and transcriptome analysis has provided important information on the controlling mechanisms of seed maturation. Mutation studies revealed that four genes: *FUSCA3* (*FUS3*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *LEAFY COTYLEDON1* (*LEC1*), and *LEC2* play crucial roles in seed maturation (Fischer et al., 1998; To et al., 2006). Excluding *LEC1*, which encodes the CBF like transcription factors, the other three genes encode plant-specific B3 domain transcription factors

(Lotan et al., 1998; Giraudat et al., 1992; Luerssen et al., 1998; Stone et al., 2001). Based on phenotypes, *fus*, *lec1* and *lec2* are characterized as *LEC* group due to their effect on cotyledon development, while, *abi3* displays distinct phenotypes such as the absence of chlorophyll degradation and reduced sensitivity to ABA (Vicente-Carbajosa and Carbonero, 2005; To et al., 2006*).* *ABI3*, *FUS3* and *LEC2* induce specific seed maturation genes. The B3 conserved domain in these three transcription factors suggests a similar regulatory mechanism on specific seed maturation genes. These three transcription factors bind directly to the seed maturation-specific genes RY motif and activate their expression, except for ABI3 which also has an ABA responsive element (Reidt et al., 2000; Kroj et al., 2003; Finkelstein et al., 2002). LEC1 is homologous to the HAP3 subunits of CBF class transcription factors and activates a specific group of genes required for normal Arabidopsis seed development (Lotan et al., 1998). In addition, LEC1 also plays an important role in early embryogenesis (Lotan et al., 1998).

Studies from a single mutation event of *FUS3*, *LEC* and *ABI3* and the additive effects of double mutations indicate these three genes work in parallel pathways (To et al., 2006). Ectopic expression studies of *LEC1* revealed that *LEC1* regulates seed storage proteins (SSPs) via the induction of *ABI3* and *FUS3* (Kagaya et al., 2005). Recently, analysis of single, double and triple maturation mutants of *LEC1*, *FUS3*, *ABI3* and *LEC3* provided more details about the interaction of these four genes and established a network of local and redundant regulation of these four genes on Arabidopsis seed maturation (To et al., 2006).

2.2 Seed Dormancy

2.2.1 Seed Dormancy

Seed dormancy is a very complex, controversial and poorly understood field in seed biology (Bewley, 1997a; Finch-Savage and Leubner-Metzger, 2006). Dormancy can be defined simply as a seed trait that blocks the germination of viable seeds under favorable conditions (Bewley, 1997a). However, this definition does not accurately describe this character and some researchers have developed more elaborate theories to define dormancy (Vleeshouwers et al., 1995). Generally, seed dormancy is considered to be an adaptive trait (Foley, 2001). Through this adaptation, seed germination occurs when conditions are suitable for both the germination and subsequent establishment of seedlings (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage et al., 2007). The degree of seed dormancy is variable in different species and is also controlled by both genetic and environmental factors (Li and Foley, 1997). Due to its importance in seed germination, it is necessary to study the induction and release of dormancy during seed development and seed germination (Vleeshouwers et al., 1995).

Seed dormancy can be divided into primary dormancy and secondary dormancy based on the dormancy state (Foley, 2001). Primary dormancy is established during seed development, specifically at a later stage of seed development. Non-dormant seeds incubated under adverse conditions may be induced into secondary dormancy (Bewley, 1997a; Gubler et al., 2005; Cadman et al., 2006). Seed dormancy can be classified as seed coat-imposed dormancy and embryo dormancy due to the different location of constraints on germination (Bewley, 1997a; Foley, 2001). By physiological classification, seed dormancy can be categorized into five groups: physiological dormancy (PD), morphological dormancy (MD), morphophysiological dormancy (MPD), physical

dormancy (PY) and combinational dormancy (PY+PD) (Baskin and Baskin, 1998, 2004; Finch-Savage et al., 2006).

2.2.2. Dormancy Induction and Release

Primary dormancy is established during seed maturation to suppress vivipary on the mother plant (Karssen et al., 1983; Hilhorst et al., 1995; Thompson et al., 2000; Kucera et al., 2005). ABA is speculated to be the major regulator of seed dormancy induction and maintenance (Koornneef et al., 2002; Finch-Savage and Leubner-Metzger, 2006). Studies on Arabidopsis revealed two peaks of ABA accumulation during seed development (Finkelstein et al., 2002). The first peak, which is of maternal origin, precedes the maturation stage and functions to prevent premature germination at the end of early embryogenesis (Karssen et al., 1983; Finkelstein et al., 2002). During the maturation stage, a second peak of ABA accumulation occurs in the embryo to induce seed dormancy (Karssen et al., 1983; Finkelstein et al., 2002). Arabidopsis mutants for ABA biosynthesis and reciprocal crosses with the wild type have provided important clues that ABA regulates the induction of seed dormancy (Karssen et al., 1983). Genetic studies on Arabidopsis and cereals revealed several QTLs which affect dormancy (Bentsink and Koornneef, 2002). From QTL analysis, a gene *DOG1* was identified in Arabidopsis that is thought to be involved in seed dormancy; however, its function is still unknown (Bentsink et al., 2006).

Several factors such as after ripening, light, temperature and certain chemicals are suggested to be involved in the breaking of seed dormancy (Finch-Savage and Leubner-Metzger, 2006; Finch-Savage et al., 2007) After ripening, a period of dry storage from a few weeks to several months relieves seed dormancy (Finch-Savage et al., 2007).

Several mechanisms are proposed to explain dormancy release by after ripening. These include fluctuating temperatures a reduction in ABA accumulation and sensitivity, a loss of light and a nitrate requirement (Finch-Savage and Leubner-Metzger, 2006). Recently, studies in tobacco and Arabidopsis seeds revealed several molecular mechanisms for the after-ripening function on dormancy release (Leubner-Metzger, 2003; Bove et al., 2005; Finch-Savage et al., 2007; Cadman et al., 2006). In tobacco, it has been shown that the expression of several genes are initiated during after ripening (Leubner-Metzger, 2003; Bove et al., 2005). Transcript analysis of Arabidopsis provided evidence that a gene program switch occurs during after ripening (Finch-Savage et al., 2007; Cadman et al., 2006). The effect of light on seed dormancy has been demonstrated in lettuce, tomato and Arabidopsis (Shinomura, 1997; Yamauchi et al., 2004) and is thought to be mediated by the reversible conversion between red (R) light which enhances germination and far red light (FR) which inhibits seed germination (Shinomura et al., 1995, 1996). This reaction is dependent on phytochromes, which represent a family of photoreceptors. Five phytochrome genes *phA*-*phE* are well characterized from studies on Arabidopsis mutants. Among these five genes, *phA* stimulates seed germination photo-irreversible after seeds are irradiated with a low fluency of light, whereas, *phB* is photo-reversible and responsible for the well know R/FR reaction on seed germination (Shinomura, 1997). Temperature, especially temperatures less than 10 ºC, is another crucial factor that releases seed dormancy. Low temperature incubation (called stratification) can relieve dormancy and promote germination of Arabidopsis seeds (Yamauchi et al., 2004). Molecular studies on the mechanism of light and low temperatures on dormancy release indicate that these two factors are integrated in part to promote seed germination by

increasing gibberellin (GA) biosynthesis or sensitivity to GA (Yamauchi et al., 2004; Finch-Savage et al., 2007). Both low temperatures and light interact to alleviate the repressive activity of two transcription factors, Spatula (SPT) and a phytochrome-interacting-factor-like5 (PIL5) (Penfield et al., 2005). However, the interaction between these transcription factors and GA biosynthesis is not very clear. Moreover, in some studies, GA is proposed to be a germination stimulator and not involved in the release of dormancy (Bewley, 1997a).

Certain chemicals such as nitrite and GAs have been suggested to be involved in dormancy release (Bethke, et al., 2006, 2007; Alboresi et al., 2005; Thomas, 1989). GAs are well known for their effects on seed germination; however, it is not known if GAs act as a dormancy releaser or germination stimulator. The role of GAs on germination will be discussed in a later section. Studies on the effect of nitrite on seed dormancy release suggest that nitrite may act as a signal and not act as a nutrient (Alboresi et al., 2005). This same study also suggests that nitrite interacts with GA and ABA to regulate dormancy release (Alboresi et al., 2005). Recently, researchers have discovered that nitric oxide (NO) acts as signal molecule involved in seed dormancy release and the effect of sodium nitroprusside, cyanide, nitrite and nitrate on seed dormancy is due to their release of nitric oxide (Bethke, et al., 2006, 2007).

2.3 Hormone Involved in Seed Germination

Plant hormones are defined as small molecular weight compounds that act as chemical messengers at low concentrations (Crozier et al., 2002). To date, mainly six groups of hormones including abscisic acid (ABA), gibberellins (GAs), ethylene, auxins, cytokinins and brassinosteroids (BRs) have been well characterized and proposed to play

crucial roles in plant development (Kucera et al., 2005). Among these hormones, ABA and GA are best known for their role in seed germination.

2.3.1 ABA and GA Effects on Seed Germination

It is well established that ABA is involved in dormancy and inhibits seed germination, while GA promotes seed germination (Bewley, 1997a; Kucera et al., 2005). The role of ABA and GA on seed germination is supported by either exogenous applications or genetic studies, primarily, via GA and ABA mutants (Karssen et al., 1983, 1989; Nambara et al., 1991; Hilhorst and Karssen, 1992; Debeaujon and Koornneef, 2000; Clerkx et al., 2003). Through the study of Arabidopsis mutants, the roles of GA and ABA in the control of seed transition from dormancy to germination were elucidated (Debeaujon and Koornneef, 2000; Parcy et al., 1994; Peng and Harberd, 2002). Several ABA biosynthetic mutants (*aba*) in Arabidopsis have reduced seed dormancy, as do ABA-insensitive mutants (*abi*) (Debeaujon and Koornneef, 2000; Clerkx et al., 2003). Conversely, intact seeds from mutants unable to produce GA in Arabidopsis (*ga1-3*) fail to break seed coat imposed dormancy and do not complete germination unless supplied with exogenous GA (Debeaujon and Koornneef, 2000). De novo GA and ABA biosynthesis during imbibition is demonstrated by the following studies: paclobutrazol, an inhibitor of GA synthesis, inhibits seed germination, in contrast to the enhanced effect of fluridone or norflurazon, which are ABA biosynthesis inhibitors (Le Page-Degivry and Garello, 1992; Debeaujon and Koornneef, 2000). GA stimulates germination in seeds where dormancy or quiescence is imposed by a wide variety of mechanisms such as incomplete embryo development, mechanically resistant seed coats, presence of germination inhibitors, and factors relating to the physiological competence of the

embryo axis (Peng and Harberd, 2002). In seeds, two different mechanisms have been proposed to explain the role of endogenous GA in the control of germination. The first one describes the induction of expression of genes encoding enzymes that hydrolyze the endosperm. This tissue confers part of the mechanical resistance to radicle protrusion, as demonstrated in tomato (Groot and Karssen, 1987), tobacco (Leubner-Metzger et al., 1996), and barley (Schuurink et al., 1992). The second mechanism consists of a direct stimulation effect on the growth potential of the embryo, as suggested for Arabidopsis (Debeaujon and Koornneef, 2000). ABA has been suggested to induce a dormant state during the later phase of seed maturation, which is overcome by GA (Debeaujon and Koornneef, 2000).

2.3.2. Metabolism of ABA and GA

The concentrations of ABA and GA are in a constant flux due to their metabolism. For example, in Arabidopsis seeds, following imbibition, ABA declines rapidly in non-dormant seeds just precceding germination, while, in dormant seeds, ABA transiently declines and then increases to the level observed in non-imbibed dormant seeds (Ali-Rachedi et al., 2004). In addition, following the release of dormancy, ABA in dormant seeds displays a similar pattern as observed in non-dormant seeds (Ali-Rachedi et al., 2004). A similar correlation between the level of ABA and seed germination ability was also observed in barley (Jacobsen et al., 2002). These studies indicate that changes in ABA following imbibition play an important role in the release of seed dormancy and subsequent germination. Molecular studies of genes involved in ABA biosynthesis and catabolism provide evidence how ABA is regulated. 9-cis Epoxycarotenoid dioxygenase (NCED) is a key enzyme controlling ABA biosynthesis (Schwartz et al., 2003; Lefebvre

et al., 2006). This enzyme (VP14) was first identified in maize and subsequently, homologous genes were identified in Arabidopsis, bean and avocado (Tan et al., 1997; Qin and Zeevaart, 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2000, 2001). Nine genes related to *NCED* were identified in Arabidopsis (Schwartz et al., 2003). *NCED3* functions mainly in stress responses; however, *NCED6* and *NCED9* which are exclusively expressed in dormant seeds, are the major genes for ABA synthesis (Lefebvre et al., 2006). Increased seed dormancy was observed in transgenic seeds overexpressing *NCED* genes (Thompson et al., 2000; Qin and Zeevaart, 2002). Several ABA catabolic pathways have been identified and categorized into two groups based on either hydroxylation (including 7′, 8′ and 9′ hydroxylation) or conjugation (Nambara and Marion-Poll, 2005; Zhou et al., 2004). 7′ hydroxylation converts ABA to 7′ OH-ABA, 8′ hydroxylation produces phaseic acid (PA) which is then converted to the stable form, dehydrophaseic acid (DPA). The conjugation pathway forms the ABA-glucose ester (ABA-GE) which is the irreversible product of ABA catabolism (Nambara and Marion-Poll, 2005). 8′ hydroxylation is proposed to be the predominant pathway for the *Brassica* species (Zhou et al., 2004). Microarray analysis in Arabidopsis revealed four genes encoding 8′ hydroxylase: *CYP707A1-4* (Kushiro et al., 2004). From functional analysis, *CYP707A2* is considered to be the major gene involved in the rapid decline of ABA during seed imbibition and dormancy release (Kushiro et al., 2004; Okamoto et al., 2006; Millar et al., 2006). In addition, expression analysis also revealed that *CYP707A1*and *2* function in seed development and *CYP707A1* and *4* play important roles in post-germination growth (Okamoto et al., 2006). Although 8′ hydroxylation is important for seed germination, other pathways may also play important roles under certain conditions.

For example, in the *etr* Arabidopsis mutant, ABA-GE is suggested to be associated with seed germination capacity (Chiwocha et al., 2005).

In contrast to the inhibitory effect of ABA on seed germination, GA is a positive regulator in seed germination. In Arabidopsis, GA_4 rapidly increases prior to radicle protrusion, indicating its role in the late stage of seed germination (Ogawa et al., 2003). Gene expression studies revealed that several GA biosynthesis genes such as *ent-kaurene oxidase* (*AtKO1*), *GA 20-oxidase* (*AtGA20ox3*), and *GA 3-oxidase1* (*AtGA3ox1*) are up-regulated during imbibition. A*tGA3ox2*, another GA 3-oxidase gene is highly associated with the last step for the production of bioactive GA_4 (Ogawa et al., 2003). A low temperature exposure (stratification) that overcomes seed dormancy and enhances seed germination increases the level of GA_4. *AtGA3ox1* is thought to be the gene responsible for this change, whereas, *AtGA3ox2* controls the biosynthesis of GA during the imbibition process (Yamauchi et al., 2004). These results indicate genes encoding GA 3-oxidases have distinct roles in GA controlled plant development (Ogawa et al., 2003; Yamauchi et al., 2004). Gibberellin 2-oxidases transform bioactive GAs into non-active forms. Among seven genes encoding *gibberellin 2-oxidases*, only *AtGA2ox2* is associated with GA inactivation during germination (Yamacuhi et al., 2007). *AtGA2ox2* mutant seeds have a high level of GA_4 which overcome the inhibitory effect of FR light on dark imbibed seed (Shinomura et al., 1996; Yamauchi et al., 2007). Isolation and characterization of genes involved in GA biosynthesis and catabolism, has led to a better understanding of how environmental conditions regulate endogenous GA levels.

2.3.3. Interaction between GA and ABA on Seed Germination

As described earlier, seed germination, in part, is regulated by the ratio of ABA to GA, which is finely adjusted by their anabolic and catabolic pathways (Koornneef et al., 1982, 1984; Seo et al., 2006). Recent studies provide some evidence about the direct interaction between these two hormones. For example, in barley, a reduction in ABA precedes GA accumulation, indicating an inhibitory effect of ABA on GA biosynthesis (Jacobsen et al., 2002). In sorghum, ABA inhibits the expression of the *GA 20-oxidase* gene, which encodes the enzyme involved in the oxidation pathway of GA biosynthesis (Pérez-Flores et al., 2003). In *aba2-2* mutant seeds, GA levels and genes related to GA biosynthesis or up-regulated by GA are enhanced compared to wild type seeds (Seo et al., 2006). In *cyp707a2-1* mutant seeds, all of these events are inhibited due to the elevated level of endogenous ABA (Seo et al., 2006). Based on the above results, we can propose that the suppressive effect of ABA on seed germination is, in part, via the inhibition of GA biosynthesis. Thermoinhibition studies of lettuce seeds revealed that high temperatures inhibit lettuce seed germination by the induction of ABA accumulation, whereas, exogenous GA overcomes this ABA imposed thermoinhibition by enhancing ABA catabolism (Gonai et al., 2004). This observation suggests that GA directly affects ABA accumulation by affecting the catabolism of ABA.

2.3.4 ABA and GA Signal Transduction

Environmental factors affect seed germination via interaction between ABA and GA; however, how seeds perceive these environmental cues and how they are transmitted is still obscure. The best known components of the GA signal transduction are a group of DELLA proteins that act as the negative regulators of the GA response (Wen and Chang,

2002; Lee et al., 2002). In Arabidopsis, five DELLA proteins, RGA (Repressor of GA1-3), GAI (GA-insensitive), RGL1 (RGA-like 1), RGL2, RGL3 were identified (Wen and Chang, 2002; Lee et al., 2002; Tyler et al., 2004). Functional analysis suggests that RGA, GAI and RGL1 are involved in vegetative growth, whereas RGL2, when combined with RGL1 is responsible for the GA response in seed germination (Wen and Chang, 2002; Lee et al., 2002; Tyler, et al., 2004). Recently, a positive gene in GA signaling transduction was isolated from a recessive GA-insensitive Arabidopsis dwarf mutant *sleepy1* (Steber et al., 1998; McGinnis et al., 2003). Gene function analysis revealed that *SLEEPY1* (*SLY1*) encodes an F-box subunit of SCF E3 ubiquitin ligase (McGinnis et al., 2003). RGA2 is degraded in the *ga1-3* mutant after GA treatment; however, high levels are found in the *sleepy1* mutant and are unaffected by GA treatment, demonstrating the requirement of SCF^{slp1} for the degradation of RGL2 (Lee et al., 2002; Tyler, et al., 2004). Studies with *ga1-3* suggest that the disappearance of RGL2 is necessary for seed germination; however, high RGL2 levels are recorded in germinated *sly1* mutant seeds (Lee et al., 2002; Tyler, et al., 2004; Ariizumi and Steber, 2007). These results show that degradation of RGL2 is not a prerequisite for *sly1* mutant seed germination. It is possible, in the *sly1* mutant, germination is achieved by inactivation of the RGL2 protein and not via degradation (Ariizumi and Steber, 2007).

Although there is considerable knowledge on GA signaling, elucidation as to how GA is perceived and its receptors are unknown. An unknown protein Os GID1, which is homologous to hormone –sensitive lipase in mammals was isolated from a GA insensitive dwarf mutant in rice (Ueguchi-Tanaka, et al., 2005). It is postulated that OsGID1 is a soluble receptor of GA signaling in rice (Ueguchi-Tanaka, et al., 2005,

2007). In Arabidopsis, three genes homologous to rice *OsGID1* were discovered by screening the database (Nakajima et al., 2006). Products of these genes have similar biochemical and physiological characters as *OsGID1,* which suggests that products of these three genes are putative GA receptors in Arabidopsis (Nakajima et al., 2006).

As discussed above, ABA is an important hormone in the regulation of seed dormancy, germination, plant growth and development. In addition, ABA is also involved in plant acclimation to several abiotic stresses such as low temperatures, drought and salinity (Finkelstein et al., 2002). Several genetic studies have provided evidence as to how ABA is involved in seed dormancy and germination. Five ABA insensitive loci *ABAI1-5* were identified by screening mutant seeds which germinated when exposed to ABA concentrations that were inhibitory to the wild type (Finkelstein and Somerville, 1990; Finkelstein, 1994; Leung and Giraudat, 1998). *ABAI1* and *ABI2* genes encode proteins homologous to serine/threonine phosphatase 2Cs (PP2Cs) (Gosti, et al., 1999; Leung et al., 1997; Leung and Giraudat 1998), while, *ABAI3*, *ABI4* and *ABA4* encode transcription factors (Leung and Giraudat, 1998). The *abi1* and *abi2* mutants are pleiotropic in that ABA sensitivity is affected in both seed and vegetative tissue (Gosti et al., 1999). It is not known unequivocally if *ABI1* and *ABI2* are involved in ABA signal transduction or if they are positive or negative regulators of ABA signaling (Leung et al., 1997; Sheen, 1998; Gosti et al., 1999). Over expression of *ABI1* in maize protoplast indicated that ABI1 is a negative regulator of ABA signaling (Sheen, 1998). The hyper ABA sensitivity of the intragenic revertants of *ABI*1 and *ABI2* further suggested that ABI1 and ABI2 act as negative regulators (Gosti et al., 1999; Merlot et al., 2001). Based on these results, two models were proposed to explain ABI1/ABI2 function

in ABA signal transduction. The first is based on a physiological response. ABA activates the enzyme activity of ABI1/ABI2 which inhibits ABA signal transduction. The second model suggests that ABA represses the catalytic activity of ABI1/ABI2 (Gosti, et al., 1999; Merlot et al., 2001). However, neither of the above models is consistent with the findings that ABA increases the activity of PP2Cs and overexpression of *ABI1* can not block ABA signal transduction (Merlot et al., 2001; Wu et al., 2003). Studies with ABA hypersensitive germination mutants identified another two PP2C proteins, AGH1-1 andAGH-3-1, which also act as negative regulators in ABA signal transduction (Nishimura et al., 2007; Yoshida et al., 2006). Expression analysis revealed that AGH1-1 and AGH3-1 may interact together for ABA signaling in seed development and germination (Nishimura et al., 2007; Yoshida et al., 2006). RNA metabolism is also suggested to be involved in ABA signal transduction. The ABA hypersensitive loci ABH1, SAD1, HYL1 and AHG2 encode components associated with RNA metabolism (Hugouvieux et al., 2001; Lu and Fedoroff, 2000; Nishimura et al., 2005; Xiong et al., 2001).

ABI3, *ABI4* and *ABI5* encode the B3-, AP2- and bZip-domain transcription factors, respectively and regulate the effect of ABA on seed maturation, dormancy and germination (Finkelstein and Lynch, 2000a; Finkelstein, 1998; Giraudat et al., 1992; Lopez-Molina and Chua, 2000). *ABI3* is involved in the expression of *LEA* genes which are essential for the acquisition of desiccation tolerance (McCourt, 1999; Parcy et al., 1994). Over-expression of the *ABI3* gene does not show the expected enhancement of ABA sensitivity (Bonetta and McCourt, 1999). This indicates that ABI3 would be a factor for the implementation of signaling, rather than an integral component of ABA

signaling (McCourt, 1999). *ABI5* is involved in an ABA-dependent growth arrest checkpoint to protect seed from drought during germination (Lopez-Molina et al., 2001). Further studies show that *ABI3* acts upstream of *ABI5* to regulate the re-induction of LEA genes to arrest embryo in a quiescent situation (Lopez-Molina et al., 2002). *ABI4* is thought to be involved in a combinational signaling network with *ABI3* and *ABI 5* to mediate specific seed ABA signal transduction (Finkelstein et al., 2002; Soderman et al., 2000). Moreover, *ABI4* determines the sensitivity of lipid breakdown to exogenous ABA in embryo (Quesada et al., 2000; Huijser et al., 2000; Penfield, et al., 2006).

ABA is involved in protein modification, transcription regulation and RNA metabolism; however, how ABA is perceived by the cell is still elusive. Although previous studies found several putative ABA receptors, they have not been verified (Finkelstein et al., 2002). Recently, a RNA binding protein FCA was isolated from barley with anti-idiotypic antibodies (Razem et al., 2006). This protein has a high affinity with ABA and interacts with ABA during the transition to flowering (Razem et al., 2006). This evidence suggests that FCA is an ABA intracellular receptor involved in RNA metabolism and flowering time control (Razem et al., 2006; Schroeder and Kuhn et al., 2006). A second important discovery suggests that a G-couple protein, GCR2, is an extracellular ABA receptor on the plasma membrane in Arabidopsis (Grill and Christmann, 2007; Liu et al., 2007). Physiological, biochemical and molecular studies support the concept that GCR2 is a receptor specifically binding to natural occurring ABA to control stomatal closure, seed germination and seed growth (Liu et al., 2007). The above results suggest that there are several ABA perception sites in plants.

2.4 Seed Germination Restricted by Testa

Seed coat conferred dormancy is an important factor that influences seed germination. The seed coat or testa is a multifunctional maternal origin organ which contributes to the embryo development and protection of seeds against adverse conditions. (Debeaujon et al., 2000). The seed coat delays seed germination because it restricts water and /or oxygen uptake or provides mechanical resistance to radicle protrusion (Debeaujon et al., 2000; Bentsink and Koornneef, 2002). The physical properties of the seed coat partially determine its effect on seed germination (Debeaujon et al., 2000).

Seed coat imposed dormancy is wide spread and common in both non endospermic and endospermic seeds. In non-endospermic species such as Arabidopsis, the seed coat and the aleurone layer (single outer layer of endosperm) act together to inhibit seed germination; however, in endospermic seeds as tomato and tobacco, both the testa and endosperm are involved (Debeaujon et al., 2000; Debeaujon and Koornneef, 2000; Bewley, 1997b; Leubner-Metzger, 2002; Kucera et al., 2005). Seed coat imposed dormancy is postulated to involve both GA and ABA (Debeaujon and Koornneef, 2000). Transparent testa (*tt*) mutant studies in Arabidopsis along with GA and ABA deficient mutants, indicated that GA is required to stimulate the growth potential of the embryo which is inhibited by ABA (Debeaujon and Koornneef, 2000). Arabidopsis testa mutants display low seed dormancy and functional analysis revealed that these mutants affect pigment accumulation (Debeaujon et al., 2000). Therefore, it is plausible that pigments change the physical properties of the testa or act as germination inhibitors (Debeaujon et al., 2000; Debeaujon and Koornneef, 2000; Nonogaki, 2006). Some seeds during germination display two events: seed coat rupture and endosperm rupture (Liu et al.,

2005; Müller et al., 2006). The endosperm is the living tissue and is triploid. Besides its nutritive function during seed germination, it also exerts a physical constraint to radicle protrusion. In addition, there is considerable evidence that ABA and GA exert important roles in endosperm imposed dormancy. In tobacco seeds, β -1, 3 glucanase (β-Glu) is induced in the micropylar endosperm in response to GA and is responsible for endosperm rupture (Petruzzelli et al., 2003). Transformation studies provided direct evidence that β-Glu promotes endosperm rupture and ABA is inhibitory to its action (Leubner-Metzger and Meins, 2000). It is hypothesized that β-Glu hydrolyzes the cell wall in the micropylar endosperm which facilitates seed germination (Wu et al., 2000; Leubner-Metzger, 2003). In addition, other cell wall weakening enzymes such as glucanase and endomannase which are located in the endosperm are also regulated by GA and ABA (Liu et al., 2005). In Arabidopsis, the aleurone layer is a restrictive force for radicle emergence (Liu et al., 2005; Müller et al., 2006). *NCED6,* a gene involved in ABA synthesis and *GA3ox2*, a GA synthesis gene are expressed exclusively in this aleurone layer, further indicating roles of ABA and GA on endosperm imposed dormancy (Lefebvre et al., 2006; Ogawa et al., 2003).

2.5 The Roles of Sugars and Lipids in Seed Germination

Carbohydrates produced from photosynthesis serve as an energy source and essential molecules for plant development (Rolland et al., 2002; Dekkers et al., 2004). Recently, it has been demonstrated that sugars acts as signal molecules to regulate genes that play pivotal roles in the plant´s life cycle (Rolland et al., 2002). For example, mannose inhibits Arabidopsis seed germination at a concentration that is not due to an osmotic effect (Pego et al., 1999). Glucose analogs and a hexokinase (HXK) inhibitor

revealed that the HXK pathway is involved in the mannose-mediated inhibition through energy depletion (Pego et al., 1999). Glucose can overcome the inhibition of mannose and can reverse the inhibitory effect of ABA of seed germination (Pego et al., 1999; Garciarrubio et al., 1997). The effect of glucose on ABA inhibition on seed germination was first interpreted to be related to a nutritional effect; however, recent evidence suggests that glucose releases ABA inhibition (Finkelstein and Lynch, 2000b; Garciarrubio et al., 1997). High concentrations of glucose inhibit seed germination by accumulating higher ABA levels. Interestingly, recent studies demonstrated that low concentrations of glucose delay Arabidopsis seed germination under non-stressful conditions, in contrast to its stimulatory effects under stressful conditions (Dekkers et al., 2004). Studies by Price et al. (2003) suggested that glucose delays seed germination by retarding the catabolism of ABA. Yuan and Wysocka-Diller (2006) reported that glucose inhibits seed germination by activating the ABA signaling pathway and at the same time inactivating the GA signaling pathway via the up-regulation of *ABI3* and *RGL2* genes, respectively.

In addition to sugar, lipids are another important group of metabolites that affect germination and seedling establishment. Lipids are converted to sugars by β-oxidation and the glyoxylate cycle (Baker et al., 2006). β-oxidation converts lipids to acyl-CoA, the initial substrate of the glyoxylate cycle, while, glyoxylate produces sugars using acyl-CoA as a substrate (Baker et al., 2006). The *cts* mutant was first identified as a locus which positively regulates germination (Russell et al., 2000; Footitt et al., 2006). CTS was identified as an ABA transporter responsible for the transport of acyl-CoA to the peroxisome for the initiation of germination (Footitt et al., 2002). These results indicate

that β-oxidation is essential for germination process prior to radicle emergence (Russell et al., 2000; Footitt et al., 2002, 2006). Transcriptome analysis of a *cts* mutant demonstrated that the second phase of seed germination is inhibited because specific genes related to GA and flavonoid biosynthesis were down-regulated (Carrera et al., 2007). Malate and isocitrate synthases are two unique enzymes in the glyoxylate cycle. Mutant analysis of these two genes indicated that glyoxylate is not essential for germination, but is involved in seedling establishment under unfavorable conditions (Cornah et al., 2004; Eastmond et al., 2000). However, citrate synthase (CSY) is required for seed germination (Pracharoenwattana et al., 2005). The above results demonstrate that lipid mobilization is essential for seed germination and seedling establishment.

2.6 The Use of Proteomic and Transcriptomic Approach to Study Germination

Gene expression patterns via proteomic and transcriptomic analysis aid in the identification of genes involved in germination. Over 74 novel proteins were identified prior to radicle emergence in imbibed Arabidopsis seeds (Gallardo et al., 2001). Studies on wild type and GA deficient mutants demonstrated several proteins involved in mobilization of stored reserves, cell cycle activity and facilitating radicle emergence are enhanced by GA (Gallardo et al., 2002). Transcriptome analysis also verified the role of GA at the gene level (Ogawa et al., 2003; Yamauchi et al., 2004). Proteomic analysis of an Arabidopsis CVi accession which displays dormancy revealed a group of proteins involved in dormancy release. In addition, the inhibitory effect of ABA on seed germination is associated with proteolysis of proteins which are required for seed germination (Chibani et al., 2006). Priming improves seed germination and synchrony, meanwhile it decreases seed longevity (Heydekker, 1973; Bruggink et al., 1999; Soeda et

al., 2005). Transcriptome analysis revealed the positive effect of priming on seed germination is due to its initiation of the seed germination program, whereas the negative effect is the loss of stress related proteins (Soeda et al., 2005).

Since its first discovery in mature dry cotton seeds (Dure and Waters, 1965), stored or long lived RNAs are found in almost all plant seed species. Stored RNAs are thought to play crucial roles in seed maturation and subsequent seed germination. Transcriptome analysis of stored RNAs in dry Arabidopsis seeds revealed that some of the stored RNAs contain the ARBRE motif (Nakabayashi et al., 2005). Previous studies based on metabolic inhibitors cycloheximide and actinomycin D suggested that early seed germination is depended on these stored RNAs for protein synthesis and de novo transcription is not necessary (Dure and Waters, 1965; Waters and Dure, 1966; Raghavan, 2000). In a further investigation, Rajjou et al. (2004) provided evidence that the germination potential is determined by stored RNAs and neosynthesis RNA improves the rate and uniformity of germination and seedling establishment.

Among these studies, a specific group of genes coding LEA proteins were found to play important roles in seed maturation and germination (Rajjou et al., 2004; Nakabayashi et al., 2005; Soeda et al., 2005). LEA proteins are a group of very hydrophilic proteins that are boiling stable (Galau et al., 1986). LEA proteins accumulate to high levels in the mid-to-late stages of seed maturation and disappear or decline following germination (Delseny et al., 2001; Grelet et al. 2005). LEA proteins are suggested to have a general protective role in desiccation tolerance during drying and are also important for seed germination under stressful conditions such as drought, low temperatures and salinity (Close, 1996; Swire-Clark and Marcotte, 1999; Xu et al., 1996).

Though their role in these conditions is not known, LEA proteins may act as cryoprotectants or in combination with sugars to form a glass state to prevent protein aggregation (Bravo et al., 2003; Wolkers et al., 2001; Goyal et al., 2005). Recent studies in pea reveal that a LEA mitochondrial protein coat and protect the inner membrane from desiccation (Tolleter et al., 2007). In spite of a large number of studies on the role of LEA proteins, their protective function is still unknown. In an in *vitro* experiment, a LEA protein from pea, maintained the activity of fumarase during the desiccation (Grelet et al., 2005). Primed seeds dried slowly have a longer longevity than primed fast dried seed (Soeda et al., 2005). A LEA gene *RAB18* was identified in the slow dried seed which was eventually degraded during subsequent seed germination and osmopriming (Soeda et al., 2005). It is conceivable that during slow drying, primed seeds are able to respnd to the gradual increase in water potential and synthesize protective LEA proteins (Soeda et al., 2005). Studies on LEA proteins may provide us with new insights related to seed longevity and the ability of seeds to germinate under adverse environmental conditions.

3. The effect of salinity, ABA, GA, ethylene and seed coat on the germination of a black seed line and a yellow seed line of canola at 8ºC

Abstract

Due to the cool spring in western Canada, seed quality is a key critical component to produce well established and vigorous seedlings. It has long been established that phytohormones regulate seed germination: abscisic acid inhibits germination whereas gibberellins enhance germination. We investigated the effects of ABA, GA, ethylene and inhibitors of these phytohormones alone or in combination on the germination rate of a black and a yellow seed canola (*Brassica napus*) imbibed at 8ºC. The effects of either saline solutions, osmotic solutions, fusicoccin or seed coat on the germination of canola seeds imbibed at 8ºC were also investigated. The two canola seed lines were very sensitive to increasing concentration of saline solutions; however, iso-osmotic solutions that reduced water potential were more inhibitory. The yellow seed line was more sensitive to these conditions than the black seed line. The seed coat (testa) was a major factor affecting the germination rate of the yellow seed line; however, GA_{4+7} overcome the inhibitory effect of the seed coat, whereas ABA exacerbated it. Fusicoccin was more stimulatory to germination than GA_{4+7}; however, unlike GA_{4+7}, it was unable to overcome the inhibitory effect of paclobutrazol, a GA biosynthesis inhibitor. Fluridone, an ABA biosynthesis inhibitor, was unable to overcome the inhibitory effects of a saline solution suggesting that the inhibitory effect was not due to elevated ABA. Ethylene, a stimulator of germination did not appear to be involved in the germination of these two lines. Controlled deterioration at 35ºC, 85% RH could be either partially or completely overcome by exogenous GA_{4+7}. This study demonstrates the role of hormones, salinity

and seed coat on the germination of canola seeds under less than ideal environmental conditions.

3.1 Introduction

Seed quality, a measure of seedling vigor, has been identified as a key critical component for the production of well-established canola (*Brassica napus*) seedlings under less than the ideal environment and soil conditions in western Canada. Seed germination is initiated by water imbibition that results in the enhancement of key enzymes involved in the catabolism of seed storage reserves. These events are under the control of the genetic make–up of the seed but more importantly by the soil conditions, the environment and seed quality (Gusta et al., 2004). Low soil temperatures, the lack of available soil moisture and salinity delay and reduce canola seedling emergence. In western Canada, it has long been established that applied fertilizer can dramatically reduce canola seed germination, particularly when the seed bed is relatively dry (Ukrainetz, 1974; Rostad et al., 1995).

It is well established that phytohormones are involved in seed germination particularly gibberellins (GAs) and abscisic acid (ABA) (Koornneef et al., 1982, 2002; Bewley and Black, 1994; Bewley, 1997; Finkelstein et al., 2002; Kucera et al., 2005). Ethylene promotes seed germination in a wide range of species (Ketring, 1997; Gianinetti et al., 2007). The use of ethylene inhibitors such as aminoethoxy vinyl glycine (AVG) has verified in some species that ethylene affects both percent of germination (Machabée and Saini, 1991; Calvo et al., 2004) and the germination rate (Gorecki et al., 1991). However, not all species respond to ethylene or the promotive effects are not very significant (Matilla, 2000). Both GA and ethylene promote endosperm rupture; however,

only GA promotes testa rupture (Groot and Karssen, 1987; Leubner-Metzger et al., 1995). In tobacco seeds, ABA delays endosperm rupture but does not affect testa rupture both of which can act as a major physical barrier to embryo enlargement and radicle emergence (Bewley and Black, 1994; Leubner-Metzger et al., 1995). There is substantial evidence on the antagonism between ABA and GA; however, only recently, it has been verified that GA can directly counteract the effect of ABA (Kucera et al., 2005).

In recent years, it has been shown that ABA modulates gene expression in response to dehydration, low temperature and salinity (Zhu, 2001; Shinozaki et al., 2003). Microarray-based analysis in response to ABA and osmotic stress have been done in numerous plants such as Arabidopsis (Seki et al., 2001), rice (Lin et al., 2003; Kawasaki et al., 2001), maize (Wang et al., 2002) and barley (Ozturk et al., 2002). Zhu (2001) reported that plants respond differently to salinity, Polyethylene glycol (PEG), dehydration and ABA. Buchanan et al. (2005) measured genome wide changes in gene expression in sorghum exposed to salinity, osmotic stress and ABA. The results demonstrated the existence of a complex gene regulatory network that differently modulates gene expression in a tissue and kinetic-specific manner in response to ABA, salinity and water deficit. Although there was an overlap in gene expression there were distinct responses to each of the stresses.

ABA is considered to be a key abiotic stress hormone (Zeevaart and Creelman, 1988). Canola seeds primed with ABA germinated earlier and showed a higher final percentage germination than un-primed seed, particularly under salt and osmotic stresses at low temperatures (Zheng et al., 1994; Gao et al., 1999). In contrast to priming, pre-

soaking wheat seeds in GA_3 increased the germination potential at moderate levels of salinity; however, ABA did not (Radi et al., 2001).

The response of canola seed to accelerated aging has been suggested to be a means of evaluating seed vigor (Elliot 2002, Patent 20040241635). Results from our laboratory indicate that GA can partially overcome the effects of accelerated aging, suggesting accelerated aging may impair GA biosynthesis (Gusta, unpublished data).

In this study we investigated the effects of salinity, osmoticum, ABA, GA, ethylene and inhibitors of these phytohormones on the germination of two lines of canola (*Brassica napus*) seeds (black line N89-53; yellow line, YN01-429) at 8ºC. N89-53 is a parental component in the pedigree of YN01-429; however, breeders prefer for YN01-429 due to its hiher oil and protein contents. In addition, the effect of ABA and GA on testa break-down was also determined. Finally, the effect of GA on accelerated aging was determined.

3.2 Materials and Methods

3.2.1 Seed Source

A black seed genotype (N89-53) and a yellow seed genotype (YN01-429) were obtained from Dr. G. Rakow, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada. All seeds were from the same year, 2003, and the same research station. Germination of all seeds at 23ºC was 97% or better and was regarded as high quality seed.

3.2.2 Germination Test

Germination analysis was carried out at 8ºC in a 9-cm Petri dish with three layers of Whatman no. 1 filter paper moistened with 4.5 mL of either distilled water or test

solutions. All of the experiments were done in 4 replications in a completely random design with 100 seeds per dish. 8°C was chosen as a representative soil temperature for Saskatchewan spring conditions. A seed was regarded as germinated when the radicle protruded the seed coat (testa).

3.2.3 Test Solutions

Saline solutions: K_2HPO_4-KH_2PO_4, pH 7.0:

A stock saline solution was made by adding 61.5 mL 1 M K_2HPO_4 to 38.5 mL 1 M KH_2PO_4 then diluted with 700 mL, 1.15 L and 2.40 L of distilled water respectively to make 120 mM, 80 mM and 40 mM saline solutions with a pH of 7.0. The osmotic potentials of these solutions are -0.85, -0.45 and -0.251 MPa, respectively.

Osmotic solutions:

Polyethylene glycol (PEG) 8000 (Sigma) was dissolved in water to make 12% (w/v), 15% (w/v), 20% (w/v) solutions that had the same osmotic potential as 40 mM, 80 mM and 120 mM saline solutions respectively. Osmotic potentials were determined with a vapor pressure osmometer (WESCOR, 550) as described in the manufacturer's instructions. The osmotic potentials of these solutions are -0.251, -0.45 and -0.85 MPa, respectively.

GA_{4+7} solutions:

GA_{4+7} (Sigma-Aldrich), 14.8 mg, was dissolved initially in 400 µL of 95% ethanol and was added dropwise during stirring to 39.6 mL of distilled water to make a 1 mM stock solution. A 25 µM GA_{4+7} solution (optimal concentration was detemined according to Gusta unpublished data) was made by diluting this stock solution.

Abscisic acid solutions:

ABA (S-abscisic acid, a generous gift from Toray Chemical, Japan), 10.6 mg, were dissolved initially in 400 μL of 95% ethanol and was added dropwise during stirring to 39.6 mL of distilled water to make a 1 mM stock solution. A 50 μM ABA (optimal concentration was detemined according to Gusta unpublished data) or a mixture of 25 μM GA_{4+7} and 50 μM ABA solutions were made by diluting the ABA and GA_{4+7} stock solutions.

Fusicoccin (FC) solutions:

Fusicoccin (FC) (Sigma-Aldrich), 27.23 mg, were dissolved initially in 400 μL of 95% ethanol and was added dropwise during stirring to 39.6 mL of distilled water to make a 1 mM stock solution. A 10 μM FC (optimal concentration was detemined according to Gusta unpublished data), or a mixture of 10 μM FC and 50 μM ABA or a mixture of 10 μM FC and 50 μM paclobutrazol solutions were made by diluting either FC, or the FC and ABA, or the FC and paclobutrazol stock solutions.

Paclobutrazol solution:

Paclobutrazol (Sigma-Aldrich), 11.75 mg, were dissolved initially into 400 μL of DMSO and was added dropwise during stirring to 1.9996 mL of distilled water to make a 200 mM stock solution. A 50 μM paclobutrazol solution (optimal concentration was detemined according to Gusta unpublished data) was made by diluting the stock solution.

Fluridone solution:

Fluridone (Sigma-Aldrich), 11.17 mg, were dissolved initially into 400 μL of DMSO and was added dropwise during stirring to 1.9996 L of distilled water to make a 200 mM stock solution. A 50 μM fluridone solution (optimal concentration was

detemined according to Gusta unpublished data) was made by diluting the stock solution.

Aminoethoxy vinyl glycine (AVG) solutions:

AVG (Sigma-Aldrich) was dissolved in distilled water to make a 1mM stock solution. 10, 25 and 50 μM AVG solutions were made by diluting the stock solution.

3.2.4 Controlled deterioration (CD) test

Seeds were held at 35°C, 85% RH for 0, 1, 2 and 3 weeks. Subsequent seed germination tests were carried out at 8ºC in 9-cm Petri dishes with three layers of Whatman no. 1 filter paper moistened with 4.5 mL of either distilled water or a 25 μM GA_{4+7} solution.

3.2.5 Data Analysis

Data analysis was performed with SAS (SAS Institute Inc., 9.1, 2002-2002) by using the repeated measurement with mixed model ($P<0.05$).

3.3 Results

The influence of the K_2HPO_4-KH_2PO_4 saline solutions and osmotic potentials generated by PEG on the germination of both of the canola genotypes is presented in Figure 3.1. In water at 8 ºC, the black seed line (Fig. 3.1.a) germinated faster than the yellow seed line (Fig. 3.1.b) ($P=0.008$). The saline and PEG solutions inhibited the germination of the yellow seed genotype to a greater extent than the black seed line (40 mM saline solution: $P=0.0169$; 12% PEG: $P=0.009$). After 7 days imbibition in the 40 mM saline solution, approximately 55% of the yellow seed line germinated, compared to 75% for the black seed line. Increasing the concentration of the saline solution to 80 mM

reduced the germination percentage after 7days to 45% and 25%, respectively for the black and yellow seed genotypes.

During the first 5 days, the black seed line had a higher germination percentage when imbibed in 40 mM saline solution compared to 12% PEG (P=0.001); however, after 7 days, the germination percentages were equivalent. At higher concentrations of saline solutions, PEG solutions were more inhibitory than saline solutions at comparable osmotic potentials. In comparing the two seed lines, the yellow seed line appears to be more sensitive to both saline and PEG solutions. For example, there was no difference in germination percentage after 7 days for the 40 mM saline solution and 12% PEG solution for the black seed line, whereas 15% of the yellow seed line germinated in the 12% PEG solution versus 50% in the 40 mM saline solution. There was a small difference in germination percentage for the two seed lines imbibed in 120 mM saline solution: 10% of the black seed line germinated versus 0% for the yellow seed line. Neither the black nor the yellow seed line germinated in 25% PEG.

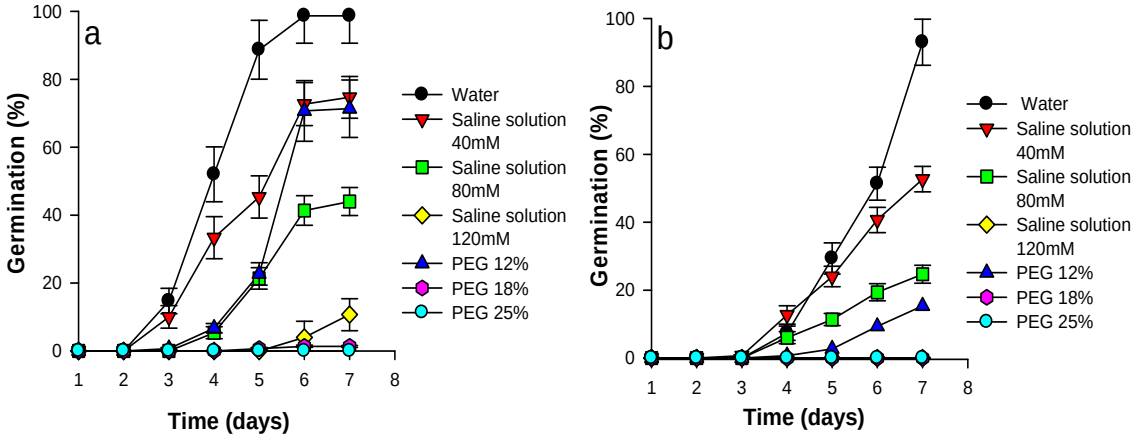


Figure 3.1. Influence of KH_2PO_4-K_2HPO_4 (saline solutions), pH 7.0 and PEG 8000 (osmoticum) on the germination of canola seeds at 8 °C. **a.** B. *napus* N89-53; **b.** B. *napus* YN01-429.
40 mM saline solution is iso-osmotic to 12% PEG, similarly, 80 mM saline solution to 18% PEG and 120 mM saline solution to 25% PEG. Values are means ± s.e. of four replicates.

The effect of GA, ABA, alone or in a combination on the germination of both lines with the seed coat on and removed is depicted in Figures 3.2 and 3.3. For seeds imbibed in water, the seed coat had no influence on the total number of seeds that germinated after 7 days, although the germination for the black seed line was higher compared to the yellow seed line. The time for 50% germination (T_{50}) in water for the black seed line was 4 days versus 6 days for the yellow seed line. If seed coat was removed, the germination was enhanced, especially for the yellow seed line. For example, if the seed coat was removed, after 4 days, 85% of the yellow seed line germinated compared to the 45% for the black seed line (P=0.006). In the case of the yellow seed line, only 15% of the seeds germinated after 4 days compared to 50% if the seed coat was removed (P=0.0032). GA enhanced the germination of both lines if the seed coat was not removed (black seed line: P=0.0356; yellow seed line: P=0.0004). The effect of GA on seed germination was less pronounced if the seed coat was removed, especially for the black seed line. ABA influenced the germination more if the seed coat was present (black seed line: P<0.0001; yellow seed line: P<0.0001); however, the total number of seeds that germinated was basically unaffected. The addition of GA_{4+7} partially overcame the inhibitory effect of ABA. The T_{50} for the yellow seed line treated with a combination of ABA and GA_{4+7} was 1.5 days shorter compared to ABA alone if the seed coat was not removed versus 0.9 days if it was removed. The difference for the black seed line was smaller: 1.2 days versus 0.6 days. Fusicoccin (FC), a diterpene glucoside, is more active than GA in stimulating the germination of both lettuce and wheat seeds (Lado et al., 1974; Marre, 1979; Ballio et al., 1981). In addition, FC almost completely removed the inhibitory effect of ABA whereas GA was only partially effective. In this study, FC was more

effective than GA in stimulating germination of the two canola seed lines (black seed line: P=0.0012; yellow seed line: P<0.0001) (Figure 3.2 compared to Figure 3.4). As shown by Lado et al. (1974), FC completely overcame the inhibitory effect of ABA (black seed line: P=0.384; yellow seed line: P=0.467), whereas, GA did not (black seed line: P=0.003; yellow seed line: P=0.0018) (Figure 3.2 compared to Figure 3.4). GA appears to play a significant role in the later stages of germination by promoting seed coat rupture (Hilhorst and Karssen, 1992). Paclobutrazol (PAC), a specific GA biosynthesis inhibitor retards radicle protrusion which suggested that seed coat rupture is affected (Karssen et al., 1989). PAC at 50 μM completely inhibited the germination of both lines (P<0.0001) (Figure 3.5); however, the addition of GA_{4+7}, completely overcame the inhibitory effect of PAC (black seed line: P=0.7842; yellow seed line: P=0.8668). FC only had a marginal effect on overcoming the inhibitory effect of PAC, suggesting its promotive effects is at a different site from GA. Fluridone (FLU), prevents the biosynthesis of ABA by inhibiting the production of a carotenoid which is a precursor for ABA synthesis (Bartels and Waston, 1978). Fluridone at 50 μM did not overcome the inhibitory effect of 80 mM saline solution (black seed line: P=0.6754; yellow seed line: P=0.5431) suggesting that ABA is not involved in the inhibitory effect of the saline solution.

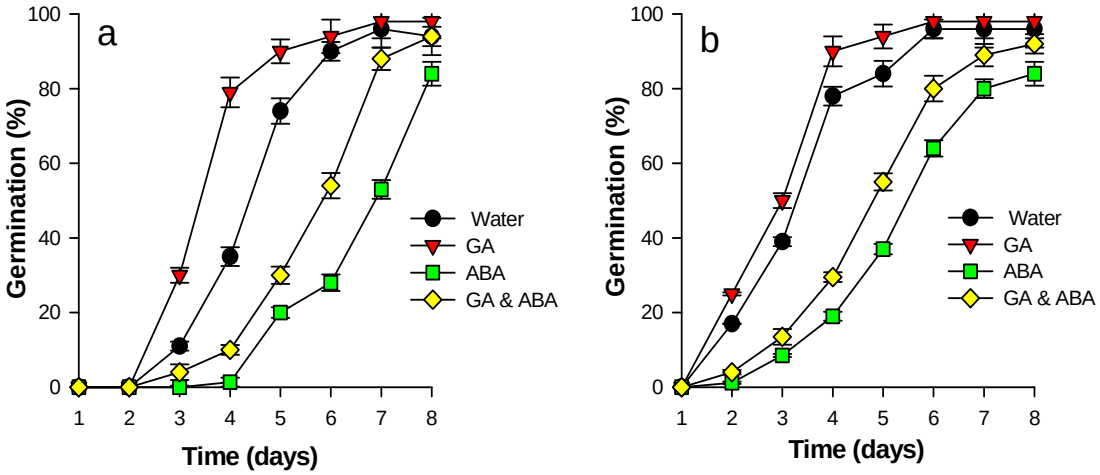


Figure 3.2. Effect of GA, ABA alone and a combination of GA and ABA and seed coat on the germination of B. *napus* N89-53 seeds imbibed at 8 °C in either 25 µM GA_{4+7}, 50 µM ABA or a mixture of 25 µM GA_{4+7} and 50 µM ABA. **a.** B. *napus* N89-53 with seed coat; **b.** B. *napus* N89-53 seed coat removed. Values are means ± s.e. of four replicates.

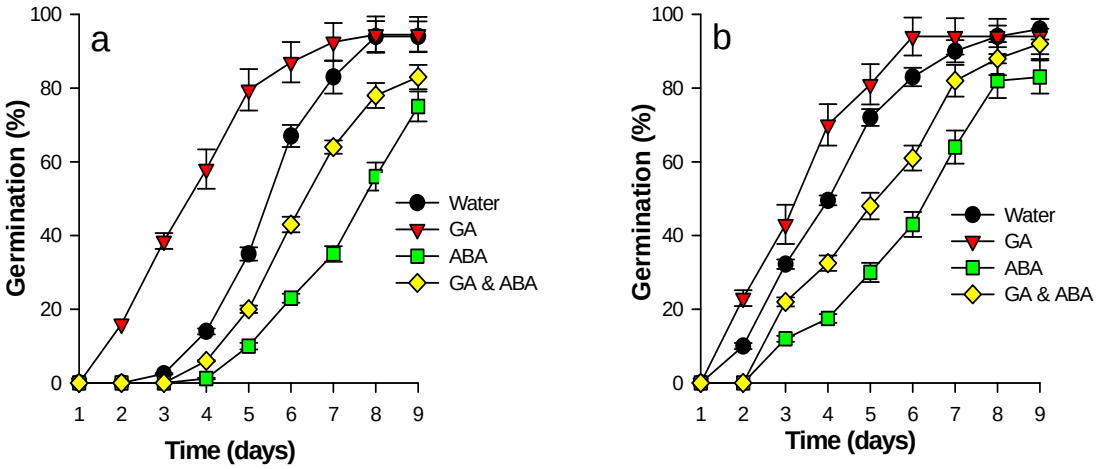


Figure 3.3. Effect of GA, ABA alone and combination of GA and ABA and seed coat on the germination of B. *napus* YN01-429 seeds imbibed at 8 °C in either 25 µM GA_{4+7}, 50 µM ABA or a mixture of 25 µM and GA_{4+7}50 µM ABA. **a.** B. *napus* YN01-429 with seed coat; **b.** B. *napus* YN01-429 seed coat removed. Values are means ± s.e. of four replicates.

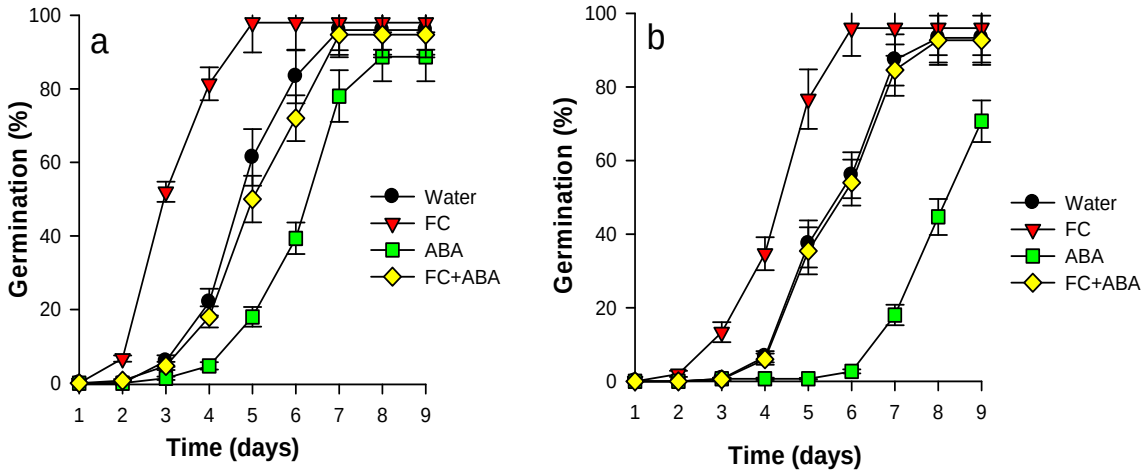


Figure 3.4. Effect of fusicoccin (FC) on the germination of canola seeds imbibed at 8 °C in either 10 µM FC, 50 µM ABA or a combination of 10 µM FC and 50 µM ABA. **a.** B. *napus* N89-53; **b.** B. *napus* YN01-429. Values are means ± s.e. of four replicates.

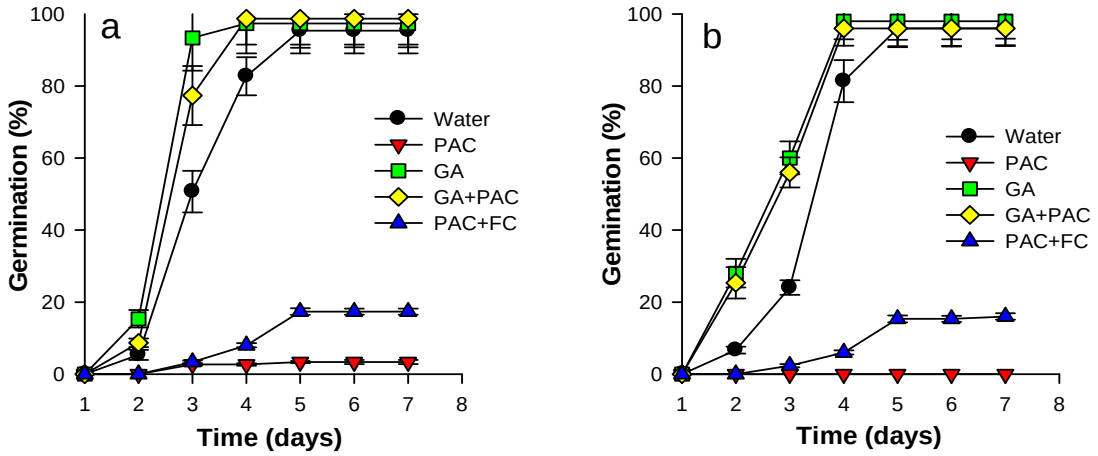


Figure 3.5. Effect of paclobutrazol (PAC) on the germination of canola seeds imbibed at 8 °C in either 50 µM PAC, 25 µM GA_{4+7}, a combination of 25 µM and GA_{4+7}50 µM PAC or a combination of 50 µM PAC and 10µM FC. **a.** B. *napus* N89-53; **b.** B. *napus* YN01-429. Values are means ± s.e. of four replicates.

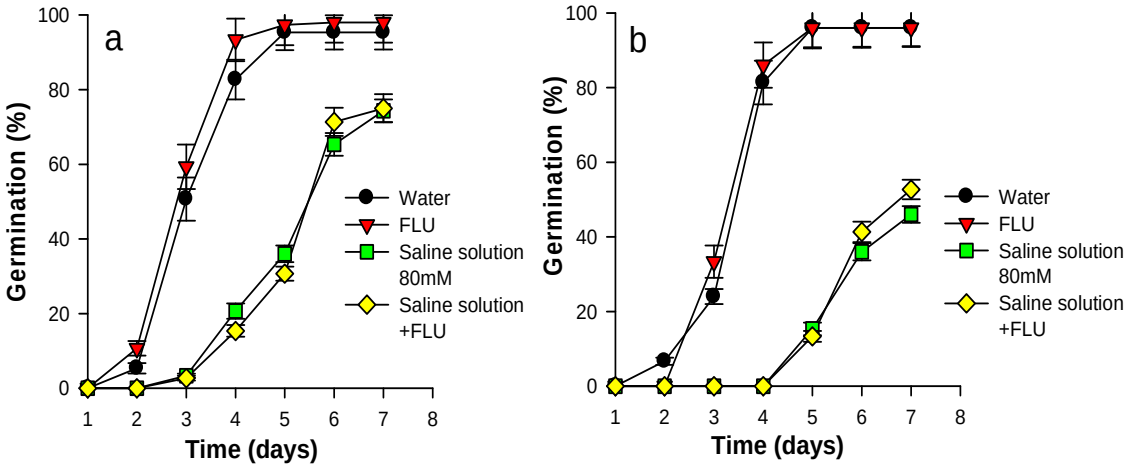


Figure 3.6. Effect of fluridone (FLU) on the germination of canola seeds imbibed at 8 °C in either 50 µM FLU, 80 mM saline solution or a combination of 80 mM saline solution and 50 µM FLU. **a.** B. *napus* N89-53; **b.** B. *napus* YN01-429. Values are means ± s.e. of four replicates.

Ethylene has been implicated in the promotion of germination by overcoming the inhibitory effect of ABA (Kucera et al., 2005). The ethylene inhibitor AVG had no effect at the concentrations tested on both lines of canola seed (at 50 µM concentration, black seed line: P=0.4075; yellow seed line: P=0.5466) (Figure 3.7).

Controlled deterioration has been suggested as a method of evaluating seed lots for seed vigor (Elliot 2002, Patent 20040241635). As shown in Figure 3.8, controlled deterioration at 35ºC and a relative humidity of 85% resulted in decreased germination to less than 50% after 2 weeks. However, the addition of GA_{4+7} to seeds subjected to two weeks of controlled deterioration completely restored the total number of germinated seeds, albeit the germination rate was reduced. Although seed germination rate was reduced (P=0.0023), the seed were still viable but impaired their germination potential. The addition of GA_{4+7} only partially overcame the effect of seeds subjected to three weeks of controlled deterioration (P=0.0085).

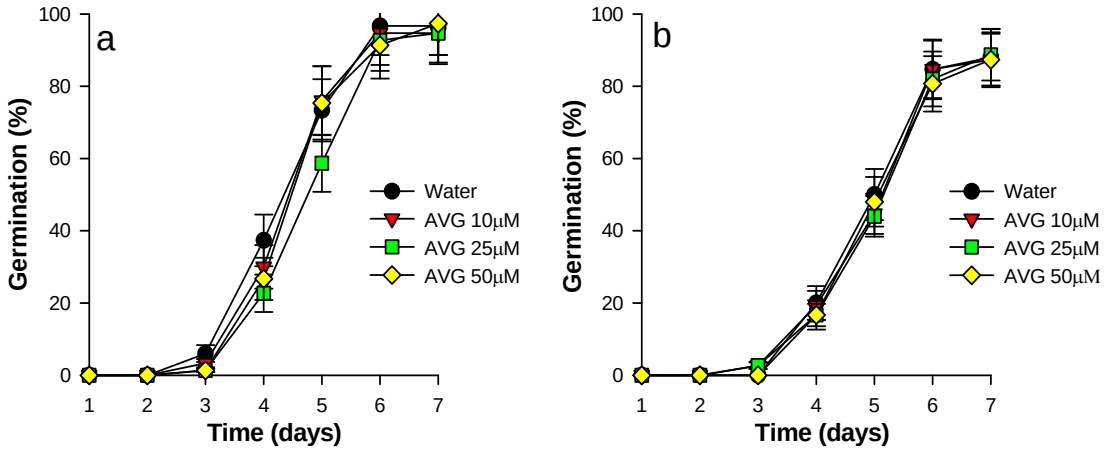


Figure 3.7. Effect of Aminoethoxyvinylglycine (AVG) on the germination of canola seeds imbibed at 8 °C in either 10 µM, 25µM or 50 µM AVG. **a.** B. *napus* N89-53; **b.** B. *napus* YN01-429. Values are means ± s.e. of four replicates.

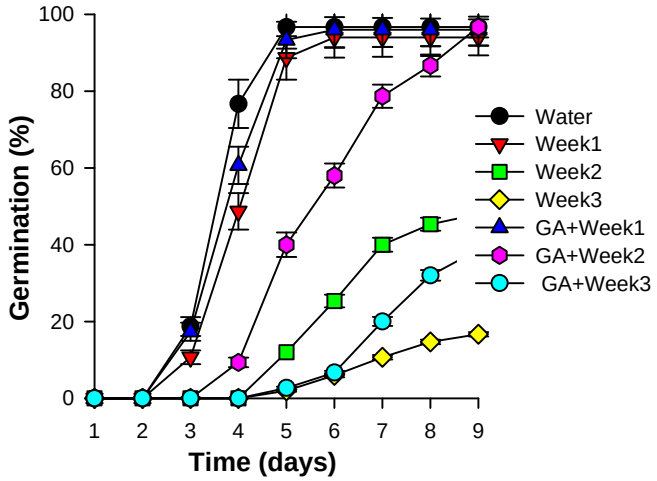


Figure 3.8. Controlled deterioration (CD) at 35 °C, 85% RH for 0, 1, 2 and 3 weeks of canola seed N89-53. Subsequent germination at 8 °C in the presence of either water or 25µM GA_{4+7}. Values are means ± s.e. of four replicates.

3.4 Discussion

A black seed canola line N89-53 and a yellow seed canola line YN01-429 were evaluated for both percent germination and germination rate at 8ºC when imbibed in

either water, GA_{4+7}, ABA, a saline solution, an osmotic solution, or inhibitors of GA, ABA or ethylene. There has been considerable interest in yellow seed canola due to its higher oil, proteins and fibre content compared to the black seed line (Rakow, personal communication; Burbulis et al., 2005). We established in this study that black seed canola line exhibits higher seed vigor at 8ºC than the yellow seed line although both lines were grown at the same location and the same year. Studies have demonstrated that the environment during seed maturation has a profound effect on seed vigor (Gusta et al., 2004; Rajjou et al., 2004; Kucera et al., 2005). A saline solution of KH_2PO_4 –K_2HPO_4 and an osmotic solution of PEG reduced the germination rate and the total number of seeds; however, solutions of PEG at the same osmotic potential as the saline solution were more inhibitory. Zheng et al. (1994) reported low soil temperatures in combination with low water potentials generated by either PEG or saline solutions reduced and delayed the germination of canola. In addition, they found that at -0.3 MPa, saline solution, NaCl, inhibited germination more than PEG. Katembe et al. (1998) working with halophyte seeds of *Atriplex species* (Chenopodiaceae) also found that NaCl solutions were more inhibitory than iso-osmotic PEG solutions. However, in cowpea and wheat, the results were the opposite (Murillo-Amador et al., 2002; Almansouri, et al., 2001). In our studies, the germination of *Brassica napus* seed was more sensitive to PEG solutions than saline solutions, indicating water uptake is critical. PEG8000 is a non-penetrating solution that restricts water uptake, while ions which can be taken up into the germplasm can either inhibit metabolism or reduce the cell water potential. This may explain why, at the same osmotic potential as the saline solution, PEG was more inhibitory.

The antagonism between GA and ABA in regard to germination is well established (Karssen et al., 1983; Karssen, 1995; Bewley, 1997; Leubner-Metzger, 2002; Kucera et al., 2005). GA appears to be promotive in the later stages of germination by controlling proteins involved in radicle protrusion and seed coat rupture (Hilhorst and Karssen, 1992; Yamaguchi et al., 1998). There is also evidence that GA is involved in cell cycle activity in the early stage of germination (Liu et al., 1994; Gallardo et al., 2002). GA is also suggested to counteract the effect of ABA by promoting ABA degradation (Kucera et al., 2005). Ethylene counteracts ABA effects and promotes endosperm rupture, but does not affect seed coat rupture, whereas ABA inhibits both the endosperm and seed coat rupture (Kucera et al., 2005). As shown in both Figures 3.2 and 3.3, GA had a significant effect on the germination rate of both seed lines imbibed at 8 ºC. There was approximately a one day difference in the T_{50} for the black seed line and a two day difference for the yellow seed line compared to their control respetively. If the seed coat was removed, the difference in T_{50} was either small or not significantly different. The seed coat is a major barrier to radicle protrusion for many seeds (Kermode, 2005), therefore it is not too surprising in canola that the seed coat is also a major barrier for the protrusion of the radicle. What is surprising is the large difference between the two lines. Therefore, it may be possible for the breeders to select lines which the testa is not a major barrier at 8 ºC. These results also demonstrate the effect of temperature on the breakdown of seed coat. Under field conditions where moisture is often limiting, this difference in time to seed coat rupture can have a profound effect on seedling establishment.

As expected, ABA inhibited seed germination, but not the percentage of germinable seeds. The yellow seed line was more sensitive to ABA than the black seed line (T_{50} 8 days versus a T_{50} 6 days respectively). The difference in T_{50} was partially attributed to the presence of the seed coat. If the seed coat was removed, the T_{50} was reduced by approximately 1.5 days. For both lines, if GA was added in combination with ABA, the inhibitory effect of ABA was partially overcome irrespective if the seed coat was present. These results suggest that ABA is involved in seed coat rupture as demonstrated by Kucera et al. (2005).

Previous studies revealed that FC is more effective than GA in overcoming the inhibitory effect of ABA (Lado et al., 1974). FC can mimic IAA and benzyladenine effetcs by enhancing and stimulating cell enlargement (Cleland, 1976, 1994). Although ABA exerts many effects, one thought is that it inhibits germination by preventing the embryo from entering and completing the growth phase, but how it prevents radicle elongation is not clear (van den Wijngaard et al., 2005). These authors suggested that FC stimulates radicle growth by up-regulating an osmo-pump protein which affects the activity of K^+ permeable ion channels in the plasma membrane. In our study, FC completely overcame the inhibitory effect of ABA, suggesting those two compounds may be acting at a similar receptor site, but different from the GA site. GA only partially overcame ABA inhibition (Figures 3.2 and 3.3); and as shown in Figure 3.5, the GA inhibitor PAC completely inhibited the germination of both lines; however GA completely overcame this inhibitory effect, FC had only a marginal effect on PAC inhibition. The finding that GA_{4+7} completely overcame PAC inhibition suggests that GA_{4+7} is a major bioactive gibberellin for canola seed germination.

The ABA inhibitor, FLU had no effect on the germination of seeds imbibed in the saline solution. Numerous studies have demonstrated increased concentrations of salinity induce a proportional increase in ABA in plants (Munns and Sharp, 1993; Cramer and Quarrie, 2002; Sharp and LeNoble, 2002) including *Brassica* species (He and Cramer, 1996). FLU has been demonstrated to decrease ABA levels in lettuce seeds subjected to supraoptimal temperatures (Yoshioka et al., 1998; Gonai et al., 2004). These authors also suggested that maintenance of high ABA levels induced by high temperatures inhibits lettuce seed germination. In tomato seeds, low temperatures and salinity have been demonstrated to elevate ABA and this could in part be the cause of reduced seed germination (Fellner and Sawhney, 2001). These results provide evidence that this phenomenon also occurs in the seed germination process. However, our results do not provide any evidence of this in canola seeds. This suggests that the inhibitory effect of the saline solution is not due to elevated levels of ABA or maybe FLU is not an effective inhibitor on ABA biosynthesis in canola.

Although ethylene is broadly implicated in promoting seed germination (Kepczynski and Kepczynska, 1997) and is antagonistic to ABA (Kucera et al., 2005), AVG, an ethylene biosynthesis inhibitor had no effect on the germination of either canola seed line. The reason for this is not known. Ethylene is known to be produced during canola seed germination (Penrose and Glick, 2001; however, it may not be essential for germination or AVG was ineffective in this study.

Resistance to accelerated ageing or controlled deterioration has been suggested by Elias and Copeland (2001) to be a method to assess canola seed quality. The mechanism by which high temperatures and high humidity reduce seed germination is not known.

Priming overcomes the inhibitory effects of NaCl and PEG solutions on canola germination (Gao et al., 2002). In this study, GA_{4+7} completely restored germination to 100% for seeds subjected to two weeks of controlled deterioration at 35 ºC with a RH of 85%. The germination rate however, was reduced compared to the control (T_{50} 5.5 days versus 3.5 days, respectively). The stimulation of germination by GA_{4+7} was greatly reduced in seeds subjected to three weeks of controlled deterioration. Therefore it appears that at the early stages of controlled deterioration, GA is either limiting or its reception sites are impaired.

3.5 Conclusion

Significant differences in the germination rate of canola seeds can be attributed to the seed coat. This difference may not be detected at warm temperatures; however, at cool temperatures, the breakdown rate of the seed coat is significant. Salinity and reduced water potential dramatically reduce the germination rate and percent of germinable seeds. Genotype difference can readily be selected following the procedure described in this study. It has been demonstrated previously that ABA and GA affect germination. We provide evidence that there is a strong interaction between ABA and GA_{4+7} on seed germination, especially at cool temperatures. There were little or no differences due to phytohormones or environmental constraints such as salinity at 23ºC (Gusta unpublished data) in contrast to profound differences observed at 8ºC. This suggests that seed vigor tests should be conducted at cool temperatures rather than warm temperatures. Seed longevity appears to be partially controlled by GA, suggesting it is potential to select for this trait.

4. Profile of plant hormones during the seed germination of *Brassica napus*

Abstract

Abscisic acid (ABA) and gibberellins (GAs) are the two major hormones that regulate seed germination in response to internal and external factors. In our study, we investigated hormone profiles in canola (*Brassica napus*) seeds that were 25%, 50 % and 75 % germinated and their un-germinated counterparts imbibed at 8ºC in either water, GA_{4+7}, a saline solution or ABA, respectively. With germination, ABA levels declined while GA_4 levels increased. Higher ABA levels appeared in un-germinated seeds compared to germinated seeds. GA_{4+7} levels were lower in seeds imbibed in the saline solution compared to seeds imbibed in water. Un-germinated seeds imbibed in ABA had lower GA_{4+7} levels compared to un-germinated seeds imbibed in water; however, the levels of GA_{4+7} were similar for germinated seeds imbibed in either water or ABA. PA and DPA increased in seeds imbibed in either water, the saline solution or ABA, while they decreased in seeds imbibed in GA_{4+7}. In addition, we found that ABA inhibited GA_4 biosynthesis, whereas, GA had no effect on ABA biosynthesis, but altered the ABA catabolism pathway. Information from our studies strongly supports the concept that the balance of hormones is a major factor controlling germination.

4.1 Introduction

Seed germination is an important process in the life history of plants and its completion sets in motion the growth of the seedling (Millar et al., 2006). Seed germination begins when a quiescent seed uptakes water and is completed with the elongation and emergence of the radicle in a turgor driven process (Bassel et al., 2004).

Germination is a very complex physiological process which is controlled by a range of developmental and external cues. Genetic and physiological studies have shown the important role played by plant hormones in regulating seed germination (Karssen et al., 1989; Jacobsen et al., 2002; Koornneef et al., 2002).

Studies on the genetic control of seed germination have mainly centered on hormone biosynthesis and hormone-responsive mutants. Through these studies, abscisic acid (ABA) and gibberellins (GAs) have been demonstrated to play an important role in the control of seed dormancy and germination. For example, in gibberellin deficient Arabidopsis and tomato mutants, the full germination response required the application of GA to the medium (Koornneef and Van Der Veen, 1980; Groot and Karssen et al., 1987). ABA-deficient (*aba*) as well as ABA-insensitive (*abi*) mutants of Arabidopsis exhibit reduced seed dormancy (Koornneef et al., 1982; Karsen et al., 1983; Jacobsen et al., 2002; Debeaujon and Koornneef, 2000; Koornneef et al., 1984), while exogenous ABA or over-production of ABA delay seed germination or enhance seed dormancy (Frey et al., 1999; Thompson et al., 2000; Lindgren et al., 2003; Nambara and Marion-Poll, 2003). Previous studies proposed that ABA induces and maintains seed dormancy (Nambara and Marion-Poll, 2003; Kucera et al., 2005), whereas GA, which is antagonistic to the effect of ABA, releases seed dormancy and promotes seed germination (Debeaujon and Koornneef, 2000; Ogawa et al., 2003; Yamauchi et al., 2004; Kucera et al., 2005). The stimulatory role of GAs on small-seeded plants such as tomato and Arabidopsis may be explained by at least two different mechanisms. First, GAs induce certain hydrolytic enzymes to overcome the mechanical resistance imposed by the endosperm and seed coat (Debeaujon and Koornneef, 2000). For example, several cell wall loosing genes which

encode β-1, 3 glucanase and endo-β-mannanase, are GA-inducible and are consistently associated with germination (Leubner-Metzger, 2002; Koornneef et al., 2002; Wu et al., 2001; Wu and Bradford, 2003; Nonogaki et al., 2000). Second, GAs increase the growth potential of the embryo as indicated in Arabidopsis (Karssen and Lacka, 1986; Debeaujon and Koornneef, 2000).

Previous studies indicated that GA-mediated developmental processes are regulated in part by changing the cellular concentration of bioactive GAs (Yamauchi et al., 2004). In barley and Arabidopsis seeds, GA increases during germination (Karssen et al., 1989; Ogawa et al., 2003; Yamauchi et al., 2004). Recent studies have shown that breaking dormancy by after–ripening, stratification, dark and smoke are strongly correlated with a decrease of ABA in seeds (Gubler et al., 2005). In addition, dormant cultivars of wheat and barley contained more ABA than non-dormant cultivars (Goldbach and Michael, 1976; Walker-Simmons and Sesing, 1990). However, some studies suggested that seed germination is determined by the concentration of ABA in imbibed seeds, and not by the concentration in dry seeds (Millar et al., 2006). For example, in dormant and non-dormant Arabidopsis seeds or embryos of barley, germination ability was highly correlated with the changing pattern of ABA upon imbibition (Ali-Rachedi et al., 2004; Millar et al., 2006). Hormone levels were shown to be strongly influenced by various endogenous and external signals (Ogawa et al., 2003; Yamauchi et al., 2004; Ali-Rachedi et al., 2004; Millar et al., 2006). Besides hormone levels, hormone sensitivity also plays an important role in seed germination. In Arabidopsis, seed germination in response to light and low temperature stimuli was identified to be due to enhanced GA sensitivity, not the amount of GA (Derkx et al., 1994).

The endogenous level of a given plant hormone is controlled by biosynthesis and catabolism. De novo GA and ABA biosynthesis during imbibition was demonstrated by the following observations: an inhibitor of GA biosynthesis, paclobutrazol, inhibits seed germination, which contrasts with the enhanced effects of fluridone or norflurazon, which are ABA biosynthesis inhibitors (Le Page-Degivry and Garello, 1992; Debeaujon and Koornneef 2000). Molecular studies indicated that two genes (*NCED6* and *9*) belonging to the Arabidopsis 9-cis-epoxycarotenoid dioxygenase (*AtNCED*) family are the major genes responsible for ABA synthesis during Arabidopsis seed development and germination (Tan et al., 2003; Lefebvre et al., 2006). At the same time, ABA 8′-hydroxylase, the key enzyme in ABA catabolism was found to be indispensable for proper control of seed dormancy and germination (Millar et al., 2006). Gene expression studies revealed that several GA biosynthesis genes such as *ent-kaurene oxidase* (*AtkO1*), *GA 20-oxidase* , *GA 3-oxidase1* (*AtGA30x1*) and *GA 3-oxidase2* (A*tGA3ox2*) are upregulated during seed imbibition and are involved in the GA control of seed germination (Perez-Flores et al., 2003; Ogawa et al., 2003; Yamauchi et al., 2004). G*ibberellin 2-oxidase* (*AtGA2ox2*) is responsible for the deactivation of bioactive GAs (Yamacuhi et al., 2007). The precise control on the expression of these genes indicates that fine-tuning of hormone levels is an important signal for plant responses to the environmental factors. Therefore, studying the hormone profiles is an invaluable tool for investigating the role played by plant hormones during seed germination.

Low temperatures and salinity are considered to be important stress factors limiting seed germination, emergence, and stand establishment, particularly for canola (*Brassica napus*), a small seeded crop. While information on the roles of hormones on the

process of seed germination has greatly increased, knowledge on their roles in seeds subjected to abiotic stress conditions is minimal.

In this study, we profiled by HPLC-ESI/MS/MS ABA, ABA metabolites, gibberellins, auxins and cytokinins during germination of canola seeds (cv. black seed line, N89-53) imbibed at 8°C in either water, 25 μM GA_{4+7}, a 80 mM buffered saline solution of K_2HPO_4-K_2HPO_4 (pH 7.0) or 50 μM ABA , when 25%, 50%, and 75% of the seeds were considered to have germinated and also the counter parts of the un-germinated seeds (75%, 50% and 25%).

4.2 Materials and Methods

4.2.1 Pant material and seed germination:

Brassica napus seeds, a black seed genotype N89-53 which was obtained from Dr. G. Rakow, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada, were imbibed at 8°C in either water, 25 μM $GA_{4+7,}$ a buffered saline solution (80 mM K_2HPO_4-KH_2PO_4, pH 7.0) or 50 μM S(+) ABA in the absence of light. All of the experiments were replicated 4 times in Petri dishes with 100 seeds per dish imbibed on filter paper with 5 mL of the above solutions.

Both germinated (25%, 50% and 75%) and un-germinated seeds (75%, 50% and 25%) were collected for hormonal analysis.

4.2.2 Extraction of plant hormones and metabolites

The extraction procedure was as described in Chiwocha et al. (2003) except that 80% isopropanol acidified with 1% glacial acetic acid was used as the extraction solution. The extraction for each sample was replicated three times.

4.2.3 Analysis of endogenous plant hormones and metabolites by HPLC-ESI/MS/MS

The following plant hormones and their metabolites were profiled for each collection: i) ABA and metabolites – ABA, phaseic acid (PA), dihydrophaseic acid (DPA), 7′-hydroxy ABA (7′-OH ABA), *neo*-phaseic acid (*neo*-PA) and ABA glucose ester (ABAGE), ii) gibberellins (GA_1, GA_3, GA_4 and GA_7, iii)auxins – indole-3-acetic acid (IAA) and indole-3-aspartate (IAAsp), and iiii) cytokinins – isopentenyladenine (2iP), isopentenyladenosine (IPA), zeatin (Z), zeatin riboside (ZR), dihydrozeatin (DHZ), dihydrozeatin riboside (DHZR) and zeatin-O-glucoside (Z-O-Glu). The precursor- to product-ion transitions and the procedure used for quantification of endogenous plant hormones and metabolites using the deuterium-labeled analogue of each compound as its internal standard were as described previously in Chiwocha et al. (2003, 2005). Each sample was injected and analyzed in triplicate by HPLC-ESI/MS/ms.

4.3 Results

4.3.1 Germination response of *Brasicca napus* seeds at 8 °C employing various incubation media:

Brassica napus seeds were imbibed at 8 °C in either water, 25 μM GA_{4+7} (GA), a 80 mM saline solution or 50 μM ABA in the absence of light. Compared to seeds imbibed in water, GA stimulated seed germination, whereas the saline solution or ABA inhibited seed germination (Figure 4.1).

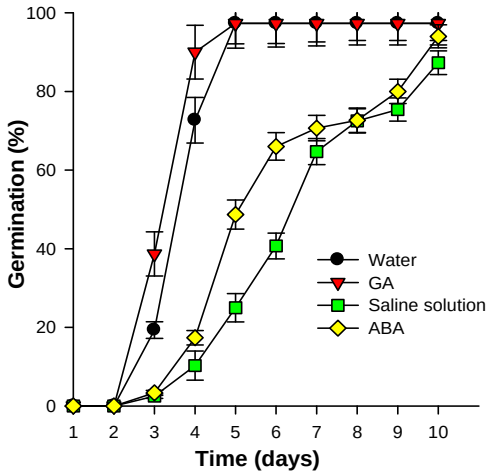


Figure 4.1. Germination profiles of *Brassica napus* (N89-53) imbibed at 8°C in either water, 25µM GA_{4+7}, a 80 mM saline solution or 50 µM ABA in the absence of light. Germination was scored as radicle emergence. Values are means ± s.e. of four replicates.

4.3.2 Hormone profiles during germination

Both germinated and un-germinated seeds were collected at 25%, 50% and 75% germination for hormonal analysis. Hormone profiles during or after germination were differentiated by this method. The role of exogenous GA_{4+7}, the saline solution or ABA on seed germination was also investigated. The differences among the endogenous hormones and their metabolites at the different stages of germination are presented.

4.3.2.1 ABA

Changes in the levels of ABA at 25%, 50% and 75% germination and their un-germinated counterparts are shown in Figure 4.2. It was not possible to determine ABA levels in seeds imbibed in exogenous ABA. However, it was possible to measure the ABA metabolites which will be discussed later (Figures 4.3 and 4.4). The level of ABA in dry seeds was 70 ng g^{-1} DW which in all cases decreased to less than 26 ng g^{-1} DW in germinated seed (Figure 4.2). A greater decrease was observed in seeds imbibed in the saline solution (20 ng g^{-1} DW or less) compared to the 26 ng g^{-1} DW for GA imbibed

seeds (Figure 4.2). In all the treatments, there was little or no change in the levels of ABA as germination proceeded. ABA levels also decreased in the un-germinated seeds but not to the extent as observed in the germinated seeds. For example, for 75% un-germinated seeds, the concentration of ABA was approximately 35 ng g^{-1} DW compared to 26 ng g^{-1} DW for 25% germinated seeds imbibed in water (Figure 4.2). Surprisingly, the ABA concentration in 75% un-germinated GA imbibed seeds was similar to the level observed in seeds imbibed in the saline solution. In all the treatments, the level of ABA was higher in the 25% un-germinated seed which was the slowest to germinate. The 50% stage is considered to have the highest rate of germination and this is reflected by the levels of ABA.

4.3.2.2 ABA metabolites

It appeared that ABA-GE was the main ABA metabolite stored in dry seeds (Figures 4.3 and 4.4). This was followed by DPA which was nearly half of the concentration as ABA-GE, then 7′ OH-ABA and finally PA. The levels of ABA-GE remained relatively constant over time in both the germinated and un-germinated seeds imbibed in water (Figure 4.3a). In contrast, there was an approximately a 40% increase in ABA-GE for seeds imbibed in GA_{4+7}, suggesting it is the major ABA catabolite (Figure 4.3b). DPA increased in seeds imbibed in water; however, it increased more in germinated seeds compared to un-germinated seeds (Figure 4.3a). In the presence of GA_{4+7}, DPA decreased in un-germinated seeds and was not detectable in germinated seeds (Figure 4.3b), suggesting a different pathway of ABA catabolism. There was a slight decrease in 7`OH-ABA in both germinated and un-geminated seeds imbibed in either water or GA, whereas, PA increased slightly (Figure 4.3).

Changes in ABA-GE in saline solution treated seeds were similar to those observed for both germinated and un-germinated seeds imbibed in water (Figure 4.4a). The increase in DPA for saline solution treated un-germinated seeds was similar to un-germinated seeds imbibed in water; however, there was 35% decrease in the germinated seeds compared to a 38% increase in the water imbibed geminated seeds (Figures 4.3a and 4.4a). The concentration of 7′OH-ABA in the saline solution treated seeds was similar to the germinated water and GA imbibed seeds and the water imbibed un-germinated seeds. In contrast to the water and GA treated seeds and the saline solution treated un-germinated seeds, there was a major increase in PA in the germinated saline solution treated seeds (Figure 4.4a). In one of the three ABA catabolytic pathways, ABA is catabolized first to PA and then DPA (Harrison and Walton, 1975). It appears that salinity affects the conversion PA to DPA. Although ABA-GE was the major ABA catabolite in germinated GA treated seeds, DPA was the major catabolite in germinated ABA treated seeds (Figures 4.3b and 4.4b). DPA increased from 120 ng g^{-1} DW in dry seeds to 155 ng g^{-1} DW in ABA imbibed seeds at 25% germination, and to over 400 ng g^{-1} DW in seeds at 50% and 75% germination. ABA-GE also increased in both germinated and un-germinated seeds imbibed in ABA; however its increase was not as large as observed for DPA (Figure 4.4b).

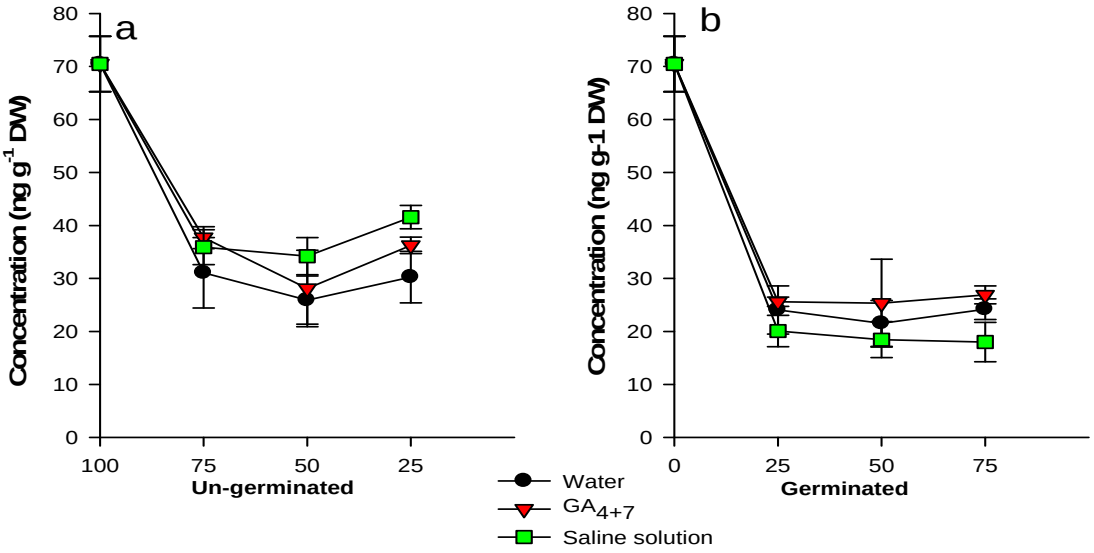


Figure 4.2. Changes in ABA in both germinated and un-germinated seeds imbibed at 8°C in either water, a saline solution or GA_{4+7} in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Un-germinated; b. Germinated. Values are means ± s.e. of three replicates.

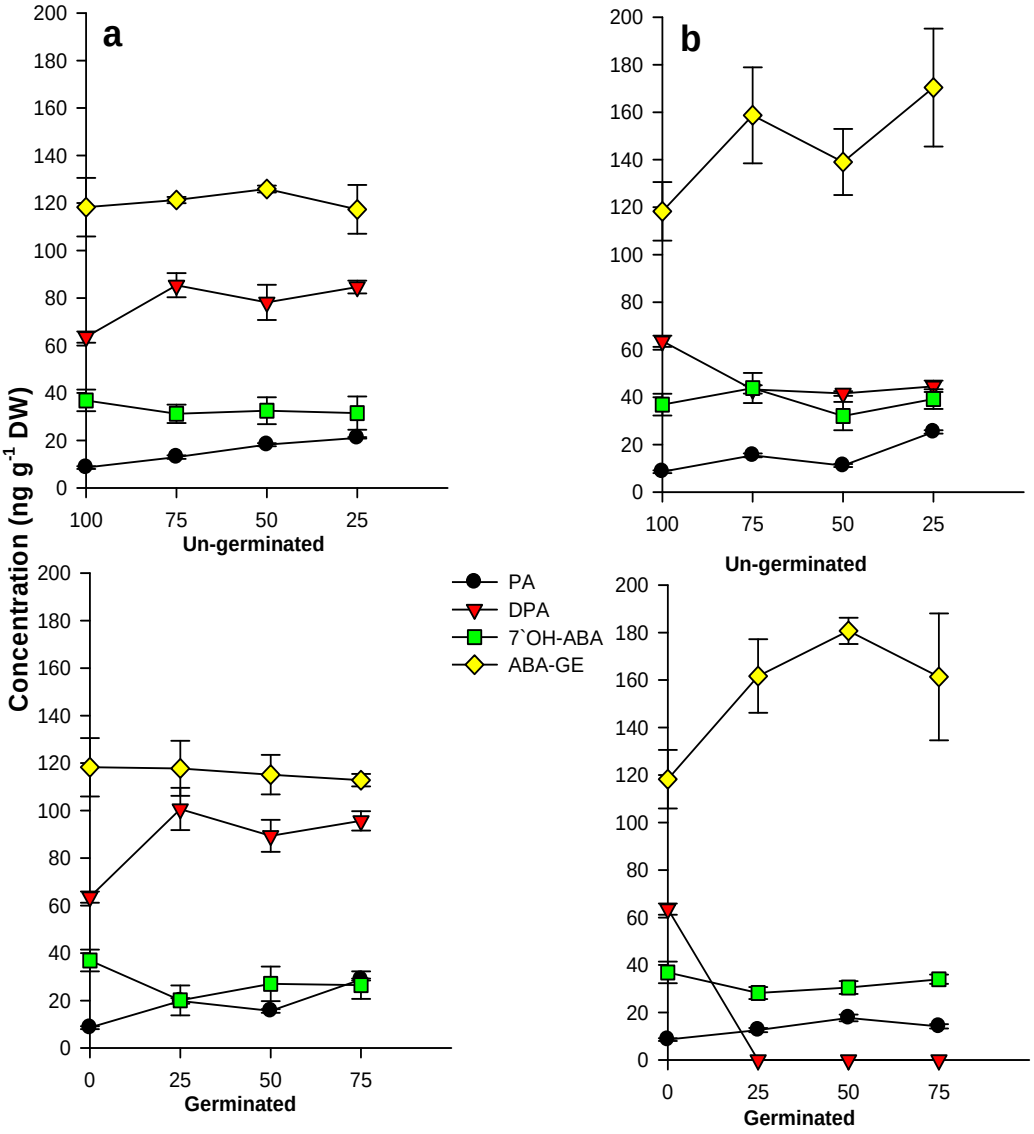


Figure 4.3. Changes in ABA metabolites in both germinated and un-germinated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Water; b. GA_{4+7}. Values are means ± s.e. of three replicates.

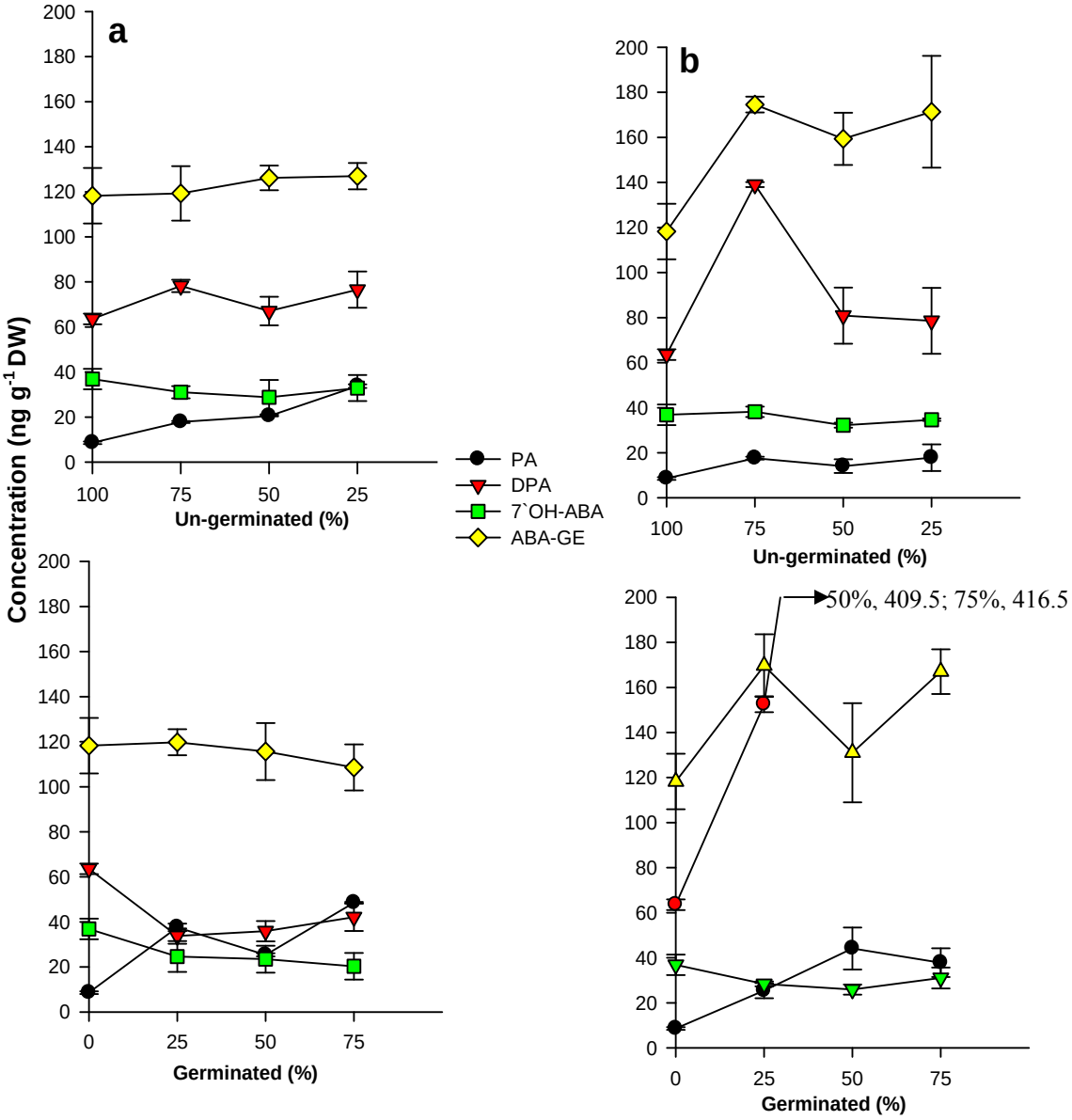


Figure 4.4. Changes in ABA metabolites in both germinated and un-germinated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Saline solution; b. ABA. Values are means ± s.e. of three replicates.

4.3.2.3 Gibberellins

GA_3 was not detected in dry seeds (Figure 4.5), but at 25% germination, GA_3 increased to approximately 50 ng g^{-1} DW in seeds imbibed in water (Figure 4.5). At 50%

germination, GA_3 decreased to approximately 35 ng g^{-1} DW and then increased to 60 ng g^{-1} DW at 75% germination (Figure 4.5). There was a significant increase in GA_3 in the 75% un-germinated seeds imbibed in water, but then GA_3 decreased from 27 ng g^{-1} DW and remained constant at 15 ng g^{-1} DW in the 50% and 25 % un-germinated seeds (Figure 4.5). Trace amounts of GA_4 (4 ng g^{-1} DW) were detected in dry seeds which increased to 18 ng g^{-1} DW when 25% of the water imbibed seeds germinated and remained relatively constant thereafter (Figure 4.5). The level of GA_4 in un-germinated seeds was similar to what was observed in germinated seeds imbibed in water at all stages of germination (Figure 4.5). In contrast to the increase in GA_3 for seeds imbibed in water, there was no increase in GA_3 in seeds imbibed in either GA_{4+7} (data not shown), the saline solution or ABA (Figure 4.6). The increase in GA_4 was less in seeds imbibed in the saline solution as compared to seeds imbibed in water (Figure 4.6a). There was a two fold decrease in GA_4 in seeds after 75% germination. A similar pattern was observed in un-germinated seeds as was observed for seeds imbibed in water, except the increase in GA_4 was approximately 47% less (Figure 4.6a). In ABA treated germinated seeds, the increase in GA_4 was similar to what was observed in water treated germinated seeds (Figure 4.6b). GA_4 increased in ABA imbibed 75% un-germinated seeds, almost to the level observed in water treated un-germinated seed (Figure 4.6b). Thereafter, GA_4 decreased and decreased 50% in 25% un-germinated seeds (Figure 4.6b). GA_1 and GA_7 were not detected in our study.

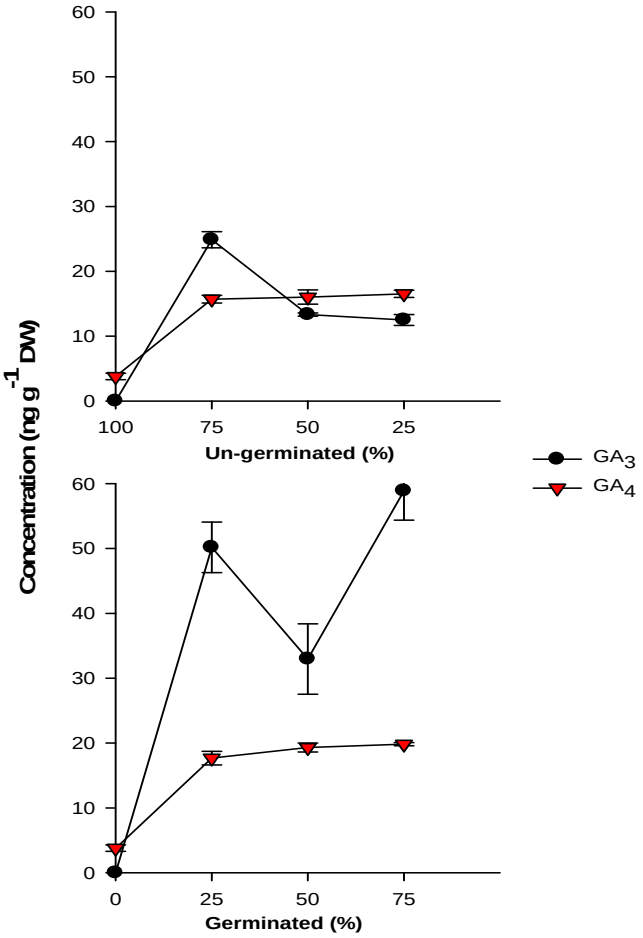


Figure 4.5. Changes in gibberellins in both germinated and un-germinated seeds imbibed at 8°C in water in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. Values are means ± s.e. of three replicates.

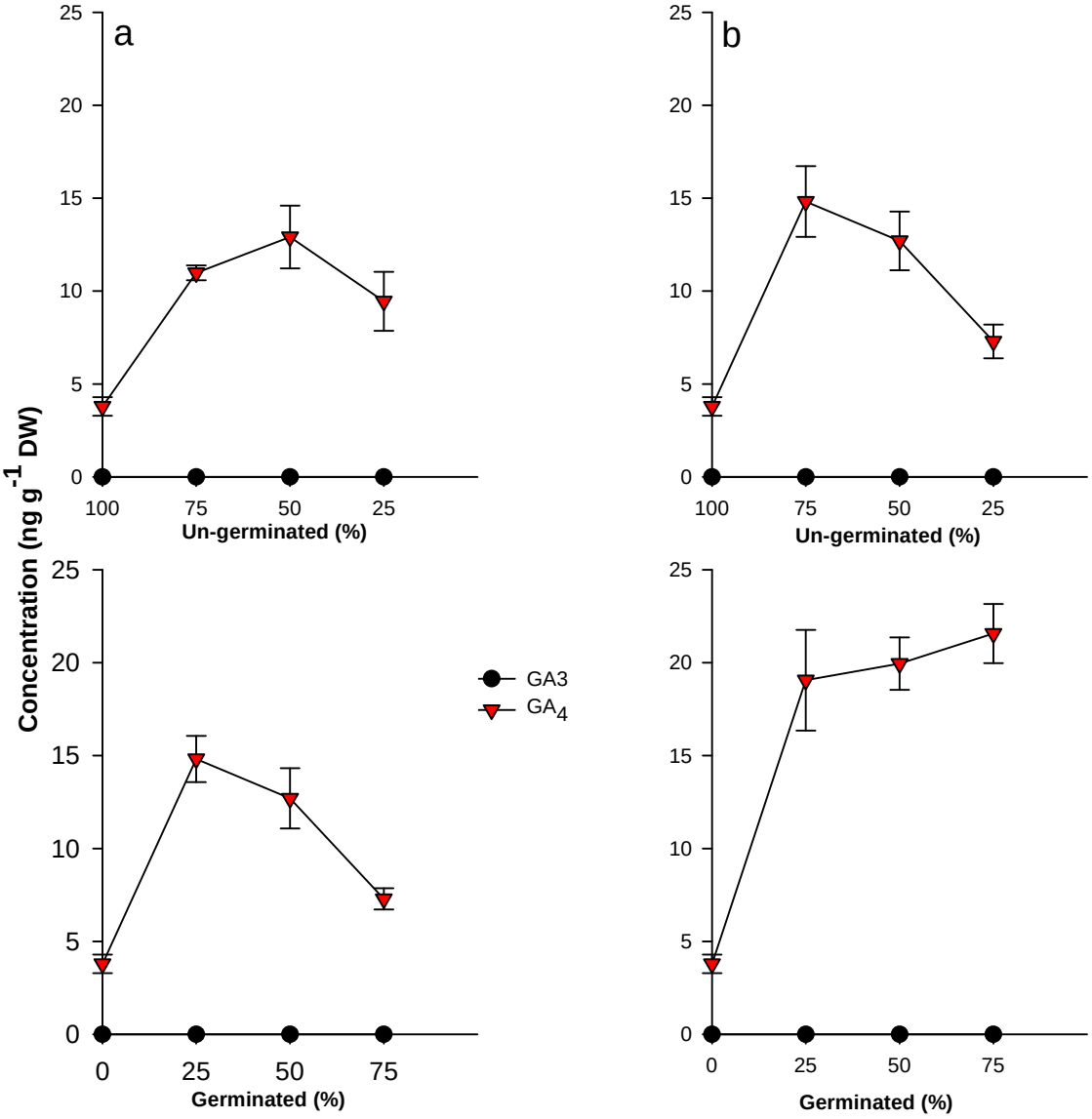


Figure 4.6. Changes in gibberellins in both germinated and un-germinated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Saline solution; b. ABA. Values are means ± s.e. of three replicates.

4.3.2.4 Auxins

There was a significant increase in IAA at all stages of germination in water; however, IAA increased more in un-germinated seeds (Figure 4.7a). IAAsp levels remained constant at all stages (Figure 4.7a). In contrast to water imbibed seeds, there

were little or no changes in either IAA or IAAsp in seeds imbibed in either GA_{4+7}, the saline solution or ABA (Figures 4.7b and 4.8).

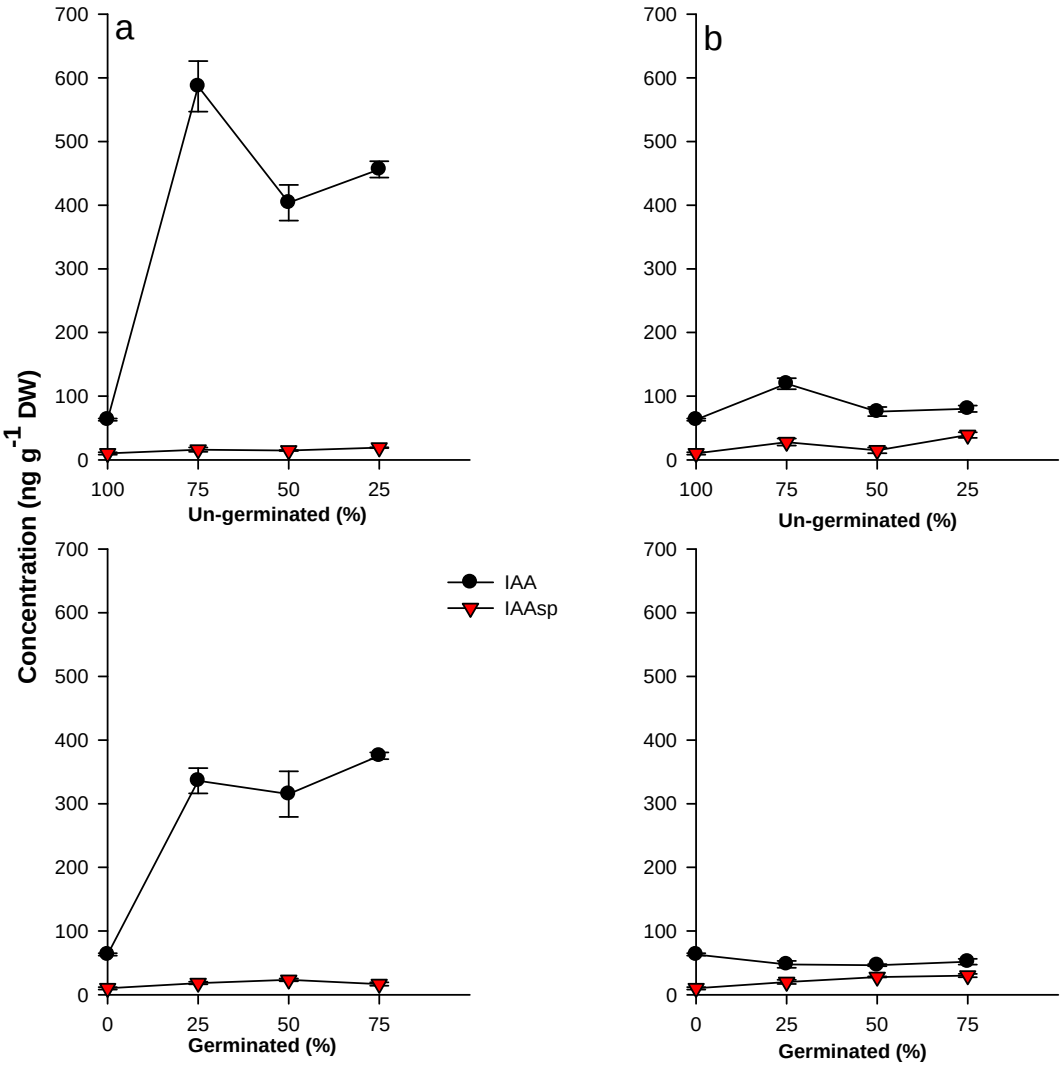


Figure 4.7. Changes in auxins in both germinated and un-germinated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Water; b. GA_{4+7}. Values are means ± s.e. of three replicates.

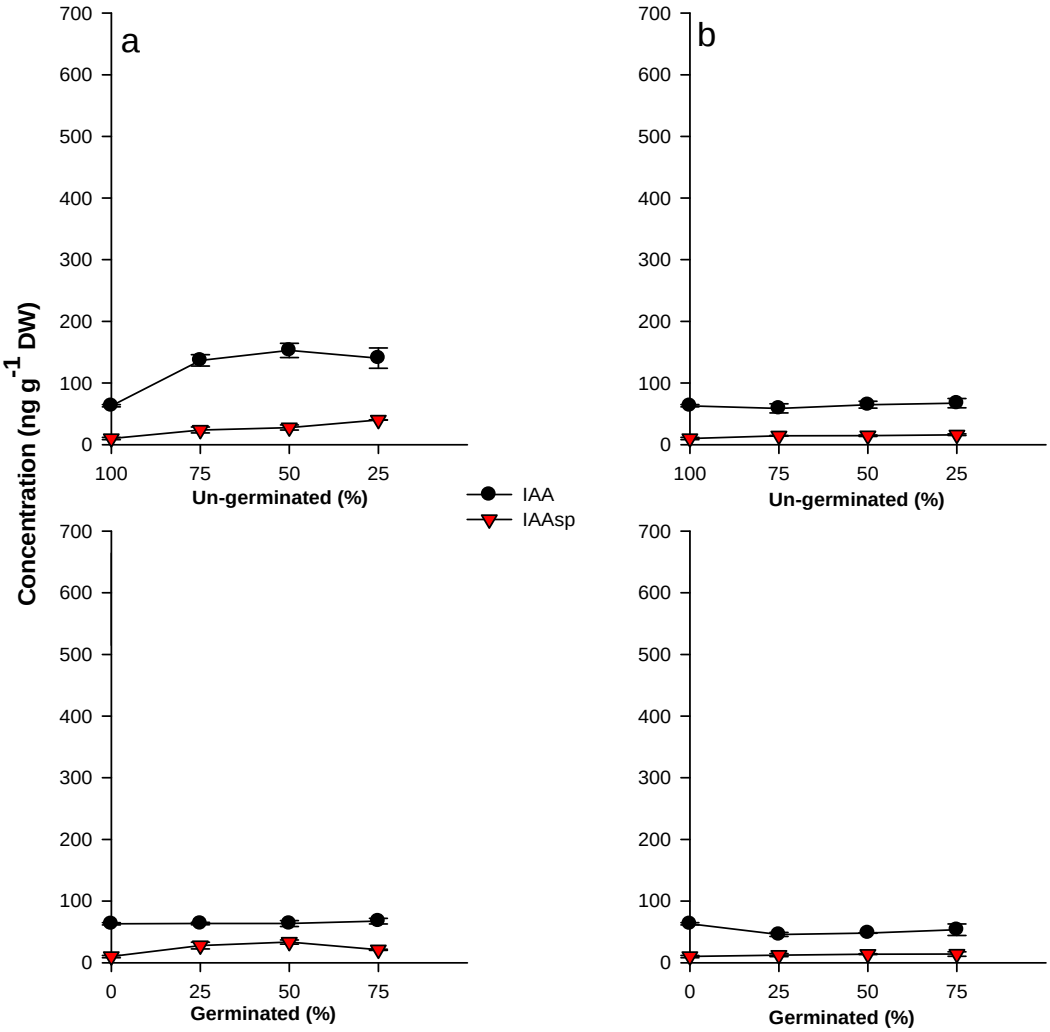


Figure 4.8. Changes in auxins in both germinated and un-germinated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Saline solution; b. ABA. Values are means ± s.e. of three replicates.

4.3.2.5 Cytokinins

ZR, iPA and DhZR were detected in either germinated or un-germinated seeds in all treatments except seeds imbibed in ABA (Figures 4.9 and 4.10). DhZR increased markedly in seeds imbibed in water, but was not detected in seeds imbibed in GA_{4+7} (Figure 4.9). Compared to dry seeds, iPA increased in seeds imbibed in either water or GA_{4+7} (Figure 4.9). The largest increase was observed in seeds imbibed in water at 75%

of germination. For seeds imbibed in the saline solution, ZR remained at the same level as in the dry seeds (Figure 4.10). The levels of DhZR increased in both germinated and un-germinated seeds imbibed in the saline solution; however, iPA was not detected in either (Figure 4.10).

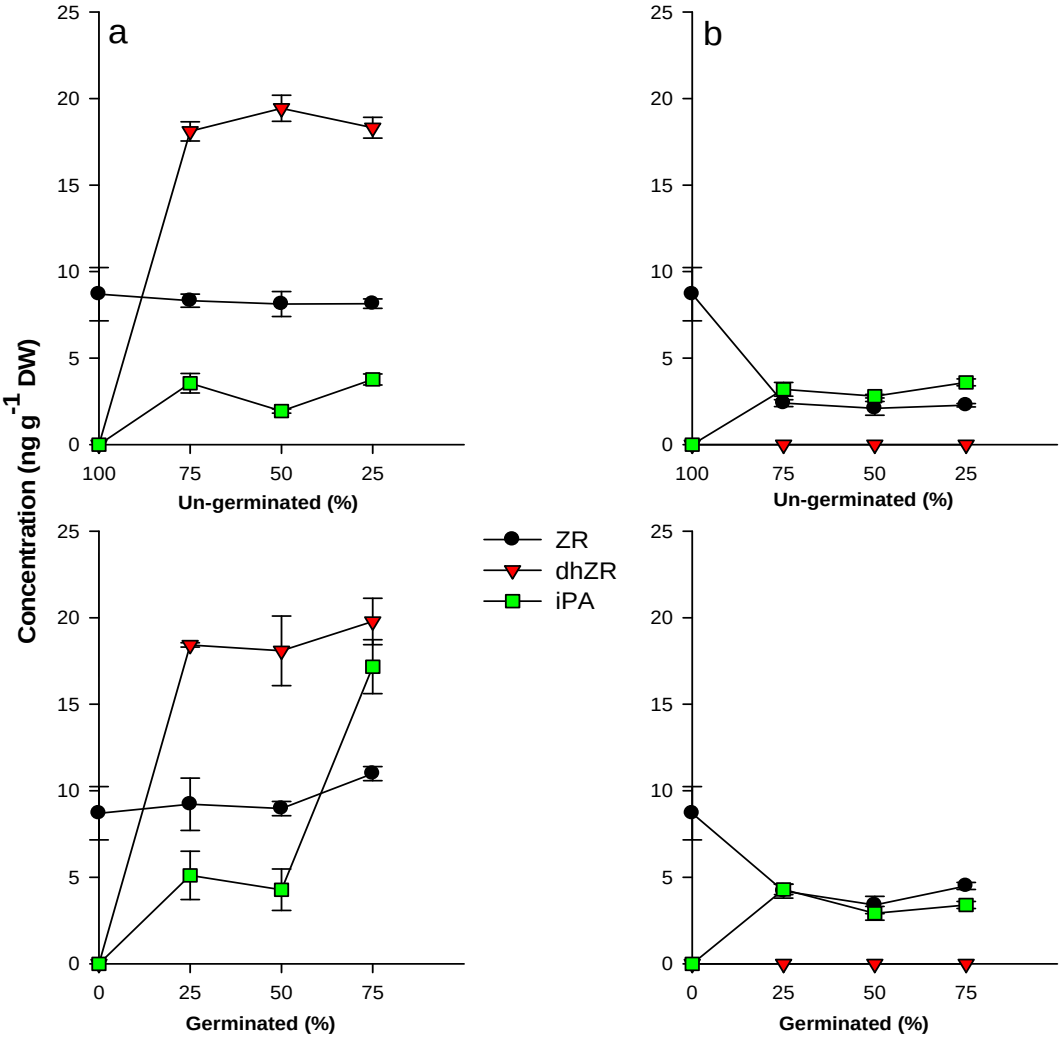


Figure 4.9. Changes in cytokinins in both germinated and un-germinated seeds imbibed at 8°C in either water or GA_{4+7} in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Water; b. GA_{4+7}. Values are means ± s.e. of three replicates.

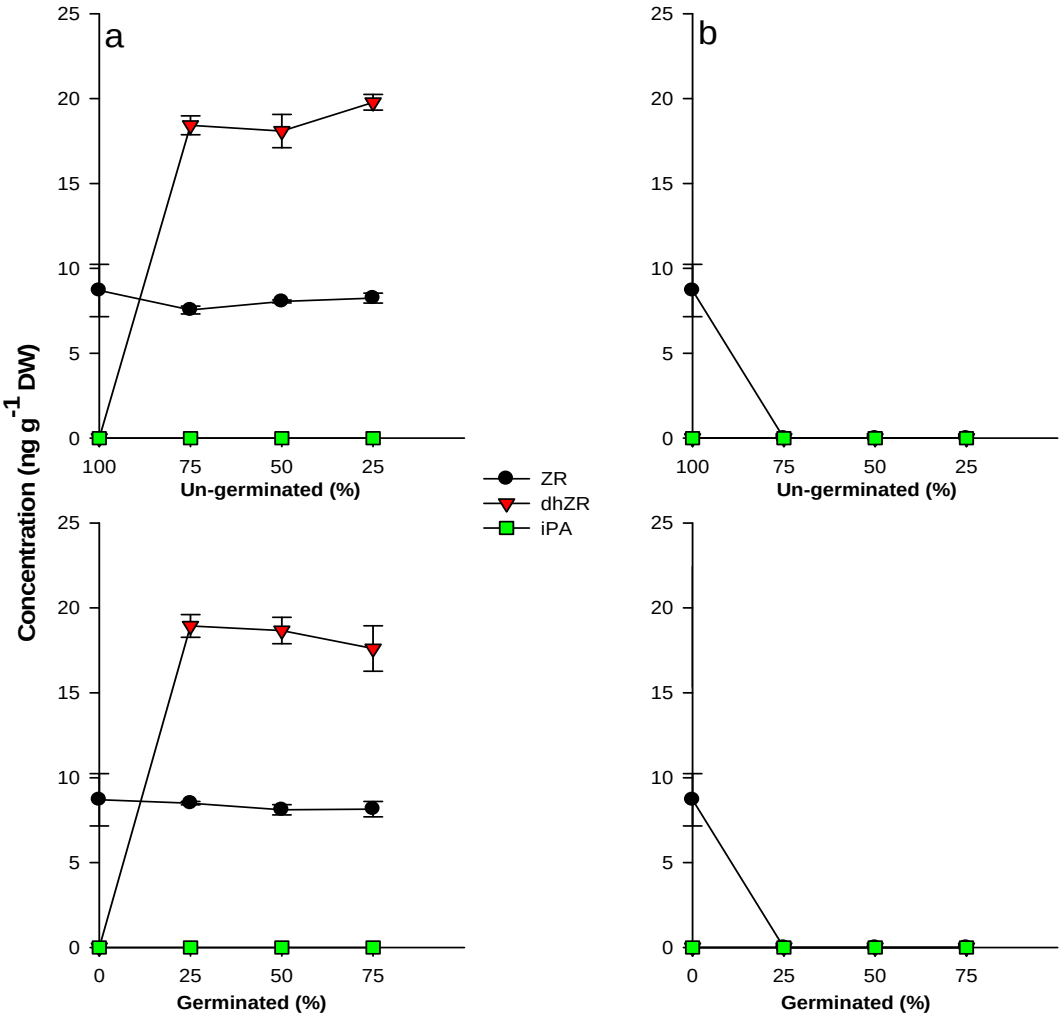


Figure 4.10. Changes in cytokinins in both germinated and un-germinated seeds imbibed at 8°C in either the saline solution or ABA in the absence of light. Seeds were collected at 0%, 25%, 50% and 75% germination and also their un-germinated counterparts at the same intervals. a. Saline solution; b. ABA. Values are means ± s.e. of three replicates.

4.4 Discussion

Compared to water, seeds germinate slightly faster in the presence of GA_{4+7} and slower when imbibed in either the saline solution or ABA (Figure 4.1). This different germination pattern is thought to be controlled by endogenous hormones such as GA or ABA. To elucidate how hormones control germination, we profiled changes in ABA and its metabolites, gibberellins (GA_3 and GA_4), auxins (IAA and IAAsp) and cytokinins

(ZR, dhZR and iPA) in both germinated seeds and un-germinated seeds at different stages of germination. In addition, we added exogenous GA_{4+7}, ABA or a saline solution to the seeds to determine what effect they had on the hormones. Profiles of ABA and its metabolites for seed imbibed in GA_{4+7} and profiles of gibberellins for seeds imbibed in ABA will be discussed in details in the section of interaction between GA and ABA.

4.4.1 ABA and its metabolism

ABA is a negative regulator in the control of seed germination (Finch-Savage & Leubner-Metzger, 2006). ABA levels in seeds are regulated by anabolism and catabolism (Okamoto et al., 2006). In our study, ABA levels decreased in all cases upon imbibition, irrespective of the treatments (Figure 4.2) which is consistent with the pattern of ABA changes observed in Arabidopsis (Ali-Rachedi et al., 2004) and barley (Millar et al., 2006). Moreover, our results reveal that un-germinated seeds contained higher ABA levels than germinated seeds in all the treatments. These results indicate that a decline in ABA is required for the initiation of germination and there is a threshold level controlling seed germination. Seeds imbibed in the saline solution germinated slower than seeds imbibed in water (Figure4.1). Compared to un-germinated seeds imbibed in water, ABA levels were higher in un-germinated seeds imbibed in the saline solution; however, they were slightly lower in germinated seeds imbibed in the saline solution. This suggests that the delay in germination for seeds imbibed in the saline solution may be a function of time required for the catabolism of ABA at 8ºC below the threshold level. This concept is supported by previous studies which demonstrated that there is a threshold level for ABA inhibition of germination (Millar et al., 2006). Higher ABA levels observed in un-germinated seeds imbibed in the saline solution may be the result of

induced ABA de novo synthesis or the delayed rate of ABA catabolism. Previous studies in lettuce (Yoshioka et al., 1998; Gonai et al., 2004), tomato (Fellner and Sawhney, 2001) demonstrated that delayed seed germination under stressful conditions is partially due to induced ABA de novo synthesis. However, fluridone, an ABA inhibitor (Yoshioka et al., 1998), had no effect on the germination of seeds imbibed in the saline solution as described in the current study (Zhang and Gusta unpublished data). The higher ABA levels observed in our study may be attributed to the rate of ABA catabolism which is inhibited by the saline solution rather than de novo ABA synthesis. Previous studies in either Arabidopsis (Ali-Rachedi et al., 2004), lettuce (Toyomasu et al., 1994) or barley (Millar et al., 2006) demonstrated a correlation between ABA levels in imbibed seeds and seed germinability. In our study, the lowest level of ABA was found in 50% un-germinated seeds followed by an increase in 25% un-germinated seeds. Seeds at 50% germination have the highest germination rate, while seeds at 75% germination have the slowest germination rate. These results provide evidence to support the concept that endogenous ABA concentration is associated with seed germinability.

Previous studies have established that ABA is catabolized through two major oxidation pathways: 8´- hydroxylation to PA and then to DPA and 7´- hydroxylation to 7´OH-ABA (Uknes and Ho, 1984; Cutler and Krochko, 1999) or a conjugation pathway to ABA-GE (Zhou et al., 2004). In Arabidopsis, molecular studies with *CYP707A1* and *CYP707A2* genes which encode two key enzymes in the ABA 8`hydroxylase pathway revealed that the 8´ hydroxylation pathway is the major pathway involved in ABA controlled germination (Kushiro et al., 2004; Okamoto et al., 2006; Millar et al., 2006). In our study, both PA and DPA increased in seeds imbibed in water as expected with the

observed reduction in ABA (Figure 4.3; however, 7´OH-ABA levels did not vary significantly in un-germinated seeds and slightly decreased in germinated seeds (Figure 4.3). ABA-GE also remained relatively constant in seeds imbibed in water although it was the major ABA catabolite in dry seeds. These results indicates that the 8` hydroxylation is the preferred pathway for ABA catabolism in canola seeds imbibed in water at 8 ºC which is consistent with previous studies. For germinated seeds imbibed in the saline solution, PA was higher compared to water imbibed germinated seeds, while DPA decreased which is in contrast to the increase observed in germinated seeds imbibed in water (Figure 4.4). These results indicate the conversion of PA to DPA in seeds is affected by the saline solution. For seeds imbibed in ABA, DPA was significantly enhanced compared to water imbibed seeds. This observation is consistent with the concept that ABA, itself, can activate its 8´ hydroxylation catabolic pathway (Uknes and Ho, 1984; Cutler and Krochko, 1999; Qin and Zeevaart, 2002). In our study, we found that ABA-GE was also greatly enhanced in seeds imbibed in ABA. It appears that the ABA-GE conjugation pathway is also activated via exogenous ABA. This is different from previous studies in plants which have shown that only the 8` hydroxylation is activated (Uknes and Ho, 1984; Cutler and Krochko, 1999; Qin and Zeevaart, 2002).

4.4.2 Gibberellins

GAs have been shown to be required for germination from studies on GA-deficient mutants (Koornneef and Van Der Veen, 1980; Groot and Karssen, 1987) and GA biosynthesis inhibitors (Karssen et al., 1989; Nambara et al., 1991). GA_4, a bioactive gibberellin increases in imbibed seeds indicating GA_4 is essential for seed germination. GA_4 may be a major bioactive gibberellin for canola seed germination at 8ºC. This

finding is consistent with studies on Arabidopsis which also demonstrated the essential role of GA_4 in seed germination (Ogawa et al., 2003; Yamauchi et al., 2004). GA_4 was lower in seeds imbibed in the saline solution compared to seeds imbibed in water. After 75% of the seeds germinated in the saline solution, the 25% un-germinated seeds had the lowest GA_4 (Figure 4.6). These results indicates that salinity delayed seed germination is partially induced by its inhibitory effect on GA_4 biosynthesis. In combination with the ABA profiles for seeds imbibed in the saline solution, we propose that the saline solution inhibits seed germination by reducing the ABA catabolism as well as GA biosynthesis.

4.4.3 GA and ABA interaction

The antagonistic roles of GA and ABA in controlling germination can occur through a direct or an indirect interaction or both. Direct actions include the interaction between their metabolism, while indirect roles would be the opposite effect of GA and ABA on genes that regulate seed germination. There is a great deal of evidence to support the indirect action of GA and ABA on seed germination. GA and ABA have opposite effects on genes encoding for endo-β-mannanase, β-1.3 glucanase, α-amylase and expansin (Leubner-Metzger et al., 1996; Leubner-Metzger, 2002; Koornneef et al., 2002; Wu et al., 2001; Wu and Bradford, 2003; Chen & Bradford, 2000). In Arabidopsis, this indirect action of GA and ABA was also shown in the expression of several ABRE-containing genes (Ogawa et al., 2003). However, there is less evidence to support the direct antagonism of ABA and GA.

GA_3 improves seed germination by reducing ABA levels in lettuce (Toyomasu et al., 1994; Gonai et al., 2004), whereas, in Arabidopsis, exogenous GA_4 had no effect on

the endogenous ABA (Ogawa et al., 2003). In our study, GA_{4+7} imbibed seeds had a higher level of ABA than seeds imbibed in water which rules out the possibility that GA affects ABA biosynthesis. Although, GA_{4+7} had no effect on ABA biosynthesis, we did observe that GA_{4+7} affected the ABA catabolic pathway compared to water imbibed seeds. In water imbibed seeds, ABA was degraded to DPA, whereas, in GA_{4+7} imbibed seeds, ABA was catabolized to ABA-GE. This finding indicates that GA alters ABA catabolism and changes it from the major 8´ hydroxylation pathway to the ABA-GE conjugation pathway. ABA-GE is assumed to be an irreversible inactive metabolite of ABA (Cutler and Krochko, 1999), although some research has suggested that it can be hydrolyzed to ABA (Sauter et al., 2002). In Arabidopsis and lettuce seeds, the ABA-GE conjugation pathway has been proposed to be the major pathway for ABA degradation (Chiwocha et al., 2003, 2005). From our studies, we found that this pathway is activated in seeds imbibed in either GA_{4+7} or ABA, whereas the 8´ hydroxylation is the preferred pathway.

In barley, it has been proposed that GA biosynthesis is inhibited by ABA (Jacobsen et al., 2002; however, no direct evidence was obtained to support this hypothesis. We observed that GA_4 levels were lower in un-germinated seeds imbibed in ABA than un-germinated seeds imbibed in water (Figure 4.6). This observation supports the hypothesis that ABA has an inhibitory effect on GA biosynthesis. Although lower level of GA_4 was detected in un-germinated seeds imbibed in ABA compared to water, GA_4 levels in germinated seeds imbibed in ABA and water were nearly identical (Figure 4.6). Based on these results, we postulate that seeds overcome exogenous ABA by accumulating GA_4. This ABA dependent GA requirement was also shown in an

Arabidopsis mutant (Debeaujon and Koornneef, 2000). Since ABA inhibits GA_4 biosynthesis, a longer imbibition time is required to attain the required level of GA to stimulate germination. Therefore, this may be one mechanism whereby ABA inhibits seed germination. In sorghum, the inhibitory effect of ABA on the expression of GA 20-oxidase gene, the crucial gene in GA biosynthesis, also support this antagonistic role of ABA on GA biosynthesis (Gonai et al., 2004).

4.4.4 Auxins and cytokinins

Auxin plays a major role in controlling cell elongation in isolated stem and coleoptile; however, there is no direct evidence that it is involved in seed germination. Only recently, a study in Arabidopsis revealed that several auxin biosynthesis genes and genes encoding auxin carrier proteins are regulated by exogenous GA_4 during seed germination (Ogawa et al., 2003). In addition, IAA level changes during seed germination were also reported in lettuce (Chiwocha et al., 2003), Arabidopsis and its *etr* mutant (Chiwocha et al., 2005). However, our study did not show a consistent involvement of auxins in canola seed germination. A similar conclusion was reached for the involvement of cytokinins. We detected ZR, DhZR and iPA during seed germination, but no association could be reached regarding their roles in germination.

4.5 Conclusion

The major hormones in controlling *Brassica napus* seed germination are ABA and GA, whereas, auxins and cytokinins had little or no effects. Reduced ABA levels and increased GA_4 contents are required for canola seed to germinate at 8ºC; however, the ratio between these two hormones may be more important. Although ABA declined in imbibed seeds in all treatments, the catabolic pathways responsible for this decline are

different. ABA inhibited GA_4 biosynthesis, whereas, GA had no effect on ABA biosynthesis; however, GA alters the ABA catabolic pathway. Both ABA catabolism and GA biosynthesis are reduced by salinity.

5. Transcriptome analysis of canola seed germination at 8ºC

Abstract

Gene expression profiles for both un-germinated and germinated canola seeds imbibed at 8ºC in the presence of exogenous hormones and a saline solution were analyzed by microarray analysis. Transcriptome analysis revealed that there are significant differences between un-germinated and germinated seeds. Seeds with different treatments are also differentiated by their gene profiles. LEA genes, hormone-related genes, hydrolase-related genes and specific seed germination-related genes were identified and their expression profiles are finely associated with seed germination performance.

5.1 Introduction

Higher plants reproduce primarily via seeds which are adapted to seasonal climates and germinate under less than ideal conditions. The vigour of seeds for economic crops is extremely important in that seeds are the starting materials for crop production and they also determine the rate and uniformity of emergence, emergence under suboptimal conditions, weed competition and yield (Gusta et al., 2004). Seed germination initiates when dry seeds imbibe water and ends when the radicle penetrates the seed coat (or testa) (Bewley, 1997a). However, seed germination is never a synchronized event. This classic germination pattern of seeds is described by a sigmoid curve that can range from 1 day to several months. Most plants, over an extended period of time, do not mature uniformly and are exposed to different degrees of salinity, heat, cold and drought. This results in differences in vigour, dormancy and the ability to tolerate abiotic stresses during storage and germination. In the late few years, there have

been many transcriptomic studies to elucidate what controls seed vigor; however, this has still remained elusive.

Hormones are well known to be involved in seed germination (Bewley and Black, 1994; Bentsink and Koornneef, 2002). ABA and GA are proposed to play antagonistic roles in the control of seed germination (Karssen et al., 1983, 1989; Hilhorst and Karssen, 1992; Kucera et al., 2005). ABA induces seed dormancy and inhibits seed germination, whereas GA breaks seed dormancy and promotes seed germination (Hilhorst and Karssen, 1992; Kucera et al., 2005). ABA and GA mutants in Arabidopsis, tomato and tobacco have proven particular valuable in providing insights on the antagonistic effect of ABA and GA on seed germination (Karssen et al., 1983, 1989; Nambara et al., 1991; Hilhorst and Karssen, 1992; Debeaujon and Koornneef, 2000; Clerkx et al., 2003). Molecular studies on ABA metabolism in germinating Arabidopsis seeds found that enzymes of the NCED family (major NCED3 and 6) (Schwartz et al., 2003; Lefebvre et al., 2006) which are responsible for ABA synthesis, and enzymes of the CYP707A family (major CYP707A1 and 2) (Kushiro et al., 2004; Okamoto et al., 2006; Millar et al., 2006) which are responsible for ABA catabolism are involved in seed germination. In elucidating the role of GA on seed germination, GA 3-oxidases 1 and 2 (Ogawa et al., 2003; Yamauchi et al., 2004), two key enzymes in GA biosynthesis and a GA 2-oxidase (Yamauchi et al., 2007), an enzyme involved in GA degradation, are crucial.

Recently, transcriptomic and proteomic studies have provided evidence that gene and protein expression patterns are switched as seeds start to germinate (Gallardo et al., 2001, 2002; Finch-Savage et al., 2007). From these studies, LEA proteins, hydrolases, kinases and hormone related genes were found to be involved in seed germination

(Gallardo et al., 2001, 2002; Soeda et al., 2005). Soeda et al. (2005) reported that gene expression, especially abiotic stress related genes that appear in seed maturation, osmopriming and germination, can be used to predict the progress of seed germination as well as the stress tolerance of seeds. From other studies, hydrolases such as endo-β-1, 3-glucanase, endo- β-mannanase are proposed to stimulate seed germination by weakening the cell wall to facilitate radicle protrusion (Wu et al., 2001; Leubner-Metzer, 2003; Petruzzelli et al., 2003). Rajjou et al. (2004) examined the effect of α-amanitin, a transcription inhibitor that targets RNA polymerase Ⅱ, on the germination of non-dormant Arabidopsis seeds and revealed that stored RNAs in dry seeds determine seed germination potential, while neosynthesized RNAs affect the rate of germination.

Low soil temperatures and salinity are considered to be major factors limiting seed germination, emergence, and stand establishment of canola in western Canada. Recently, it has been established that fertilizer and its site of application have a dramatic effect on seed germination and stand establishment. While information about the control of seed germination has greatly increased, knowledge about the response of genes and proteins for seeds germinated under abiotic stress conditions is minimal. An understanding of regulatory roles of genes and proteins on seed germination could be used to predict the seed germination and used by plant breeders to select superior genotypes.

In this report, we analyzed gene expression profiles in canola seeds (*Brassica napus*) imbibed at 8ºC in either water, GA_{4+7}, a saline solution generated by a mixture of KH_2PO_4-K_2HPO_4 or ABA. Profiles of transcriptome in seeds imbibed in these treatments provide important information on how seed germination is controlled under adverse conditions.

5.2 Materials and Methods

5.2.1 Plant material and seed germination

Canola seeds (*Brassica napus*), a black seed genotype(N89-53) which was obtained from Dr. G. Rakow, Agriculture and Agri-Food Canada, Saskatoon, SK, Canada, were imbibed at 8°C in either in water , 25 μM GA_{4+7}, a buffered saline solution (80 mM K_2HPO_4-KH_2PO_4, pH 7.0) or 50 μM S(+) ABA in the absence of light. All of the experiments were replicated 4 times in a completely random design in Petri dishes with 100 seeds per dish imbibed on filter paper with 5 mL of the above solutions. 8°C was chosen as a representative soil temperature for Saskatchewan spring conditions.

When the treated seeds reached 50% germination, both the germinated and un-germinated seeds were collected respectively for microarray analysis.

5.2.2 Microarray analysis

5.2.2.1. RNA extraction

RNA was extracted by the lithium chloride (LiCl) method developed by Vicient and Delseny (1999). Seeds were finely ground in a mortar and pestle with liquid nitrogen and were suspended in 8 M LiCl to precipitate RNA on ice overnight at 4ºC. After centrifuging at 13,000g through a QiaShredder (Qiagen) column, the pellet was dissolved in the solubilization buffer (0.5% (w/v) SDS, 100 mM NaCl, 25 mM EDTA, 10 mM Tris–HCl, pH 7.6, 2% (v/v) β-mercaptoethanol). The extraction was treated twice with an equal volume of phenol and once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1). The supernatant was precipitated with an equal volume of 4 M LiCl on ice overnight at 4ºC. After centrifuging at 13,000g for 30 min, the pellet was washed with 1 mL of 75% ethanol and dissolved in 40 μL of DEPC-water. DNA was removed from

the RNA by the DNAse kit (United Bioinformatic Inc.) as described by the manufacturer's instructions.

5.2.2.2 RNA amplification (aRNA) and labeling

RNA samples were converted to cDNA and amplified by the Ambion's AminoAllyl MessageAmp II aRNA amplification kit and labeled and purified by the CyDye Post-labeling reactive dye pack (Amersham Bioscience) according to the manufacturer's instructions.

5.2.2.3 Pre-hybridization and washing

Brassica napus 15 K cDNA microarray slides with 15,000 ESTs were obtained from Dr. Andrew Sharpe, Agriculture and Agri-Food Canada, Saskatoon, SK. Slides were incubated in the pre-hybridization buffer (5× SSC, 0.1% (v/v) SDS, 0.1 mg/mL BSA) for 45 min at 37ºC. Pre-hybridized slides were washed three times by 0.1× SSC and dried by centrifugation at1,000g for 2 min.

5.2.2.4 Hybridization and post-hybridization washing

The mixture of 11 μL labeled aRNA and 47 μL hybridization buffer (25% formamide, 5x SSC, 0.1% SDS and 0.1 mg/mL sonicated salmon) was denatured at 95°C for 3 min. The hybridization buffer was applied to the slides at 37 ºC for 12-16 hour. After hybridization, each slide was washed with by 2× SSC/0.1% SDS once at 37°C for 7 min, twice with 1× SSC at room temperature for 2 min, and then twice with 1× SSC at room temperature for 1 min. Slides were dried by centrifugation at1,000g for 2 min.

5.2.2.5 Data analysis and quantification

Hybridized slides were scanned by a ScanArray 4000 laser scanner at a resolution of 10 μm. The image analysis and signal quantification were done with Quantarray (GSI

Lumonics, Oxnard, CA, USA). Signals showing a signal value <50 in both Cy3 and Cy5 channels were eliminated from the analysis. Data storage, preliminary data processing, and Lowess normalization were performed with the Bioarray Software Environment (BASE). Further analysis was done by GeneSpring GX (Silicon Genetics). Gene expression with a difference greater than 2.5 fold compared to the control were selected. Two replicates were done for each seed sample.

5.2.2.6 Northern blot Analysis

Probes were obtained by RT-PCR with the following primers:F:5′-GTTGCGGC-TCGGCTCCAGTT-3′; R: 5′-GGCAAGTCCCAGTCCCAGAAAAGA-3′ for Isocitrate lyase (IL); F:-5′GGCGGCTCAGATTCCCATAAG-3′; R: 5′-TCTCCGTCCAGCT-CCACTCCATAC-3′ for Malate synthase (MS). Fragments of two LEA genes were isolated by the degenerate primers: F: 5′-GTCGACGARTACGGYAACCC-3′; R: 5′-CCRGGMAGYTTCTCYTTRCT-3′ as described by Porcel et al. (2005) for $D_1$400; Y: 5′-CCGATGCATACTGACG-ANWANGGNAAY-3′; K: 5′-TATGATGTCCAGG-CAGCTTCTCYTTDAT-3′ (Saavedra et al., 2006) for $D_2$600. PCR condition: 94ºC, 3 min; 94ºC, 30 Sec; 50 ºC, 1 min; 72 ºC, 1 min; 30 cycle; 72ºC 15 min, keep at 4ºC.

Total RNA (20 μg) was loaded for each sample and northern blot analysis was done as described by Gao et al. (1999). RNA transferred Hybond-N (Amersham) nylon-membranes were pre-hybridized in 1%(v/v) SDS, 2× Denhardt's solution, 50% deionized formamide and 50 μg/mL of sonicated herring sperm DNA for 4–6 h at 42ºC. Membranes were hybridized in 10% dextran sulfate, 1% (v/v) SDS, 2× Denhardt's solution, 50% deionized formamide and probes labeled by the ^{32}P-dCTP with the Random Primers

Labeling System Kit (Invitrogen) for 12-16 hours at 42 ºC. Membranes were washed and developed as described by Gao et al. (1999).

5.3 Results

5.3.1 Germination response of *Brasicca napus* seeds at 8 °C imbibed in either water, GA_{4+7}, a saline solution or ABA

The germination result for *Brassica napus* seeds imbibed at 8 °C in either water, 25 µM GA_{4+7}, 80 mM saline solution or 50 µM ABA in the absence of light is shown in Figure 5.1. Compared to water, GA stimulated seed germination, whereas ABA and the saline solution were inhibitory (Figure 5.1). GA_{4+7} only marginally increased the germination rate compared to water (T_{50} 3.5 days), whereas, both ABA (T_{50} 5.5 days) and the saline solution (T_{50} 6.6 days) significantly delayed seed germination.

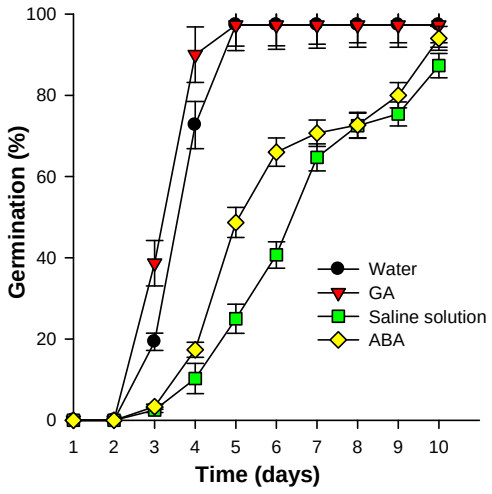


Figure 5.1. Germination profiles of *Brassica napus* N89-53 seeds imbibed in either water, 25 µM GA_{4+7}, a 80 mM saline solution or 50 µM ABA at 8°C in the absence of light Germination was scored as radicle emergence. Values are means ± s.e. of three replicates.

5.3.2 Gene expression analysis

Gene expression was done at 50 percent germination for all treatments. Un-germinated and germinated seed were analyzed separately for each of the following treatments: water, GA_{4+7}, a saline solution and ABA (listed in Table 5.1.).

Table 5.1. Seed treatments and physiological states

Treatments	Physiological states
WN	Water, 50% un-germinated seeds
WG	Water , 50% germinated seeds
GN	GA_{4+7}, 50% un-germinated seeds
GG	GA_{4+7}, 50% germinated seeds
SN	a saline solution, 50% un-germinated seeds
SG	a saline solution, 50% germinated seeds
AN	ABA, 50% un-germinated seeds
AG	ABA, 50% germinated seeds

5.3.3 Principle component analysis (PCA) of gene expression analysis for seeds imbibed in either water, GA_{4+7}, a saline solution or ABA

Principle component analysis was used to analyze the gene expression profiles of un-germinated and germinated seeds for all treatments. On the X-axis, GG, WG, GN, WN and SG grouped together, indicating similarity, whereas SN and AG grouped together and AN was in a group by itself (Figure 5.2). All of germinated seeds grouped separately from un-germinated seeds as presented with the Y-axis. In comparing germinated seeds to un-germinated seeds, the expression profiles of GG and GN were the closest followed by WG and WN which was more similar than SG and SN. The expression profiles of AG and AN were the least similar. The hierarchical clustering of genes with expression differences greater than 2.5 fold compared to dry seeds is shown in Figure 5.3. There were distinct differences in gene expression for the treatments and between germinated and un-germinated seeds. Gene expression was very similar in both germinated and un-germinated seeds imbibe in either water or GA_{4+7}, whereas the greater

differences between the germinated and un-germinated seeds were observed in seeds imbibed in either the saline solution or ABA. These results are well consistent with PCA analysis.

Figure 5.2. Principle component analysis of gene expression for un-germinated and germinated seeds imbibed in either water, GA_{4+7}, a saline solution or ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA

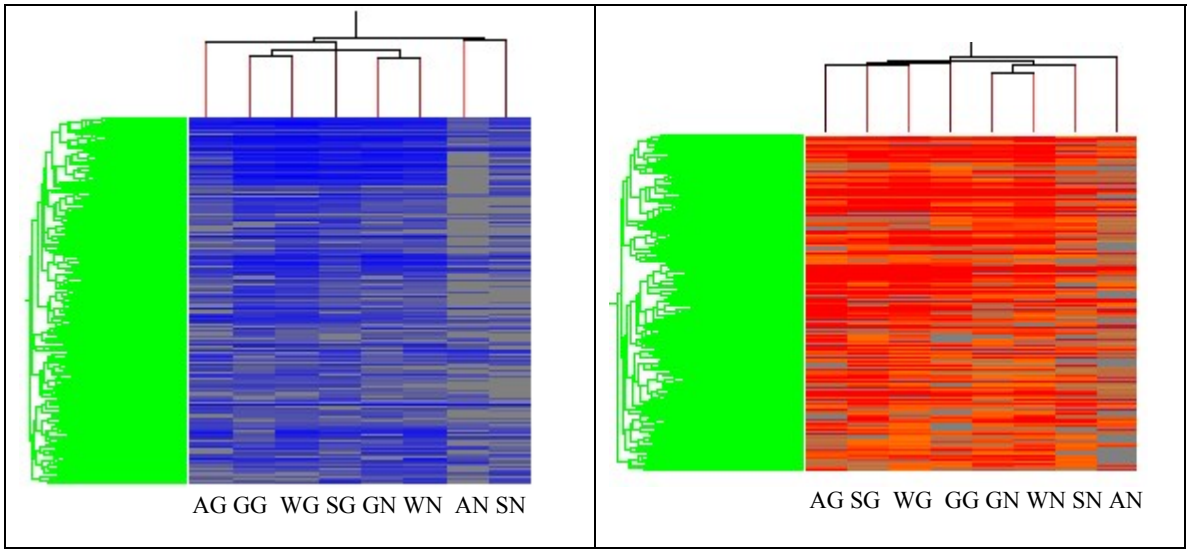


Figure 5.3. Hierarchical clustering of genes with expression differences greater than 2.5 fold compared to dry seeds. Blue color represent genes down regulated; red color, represent genes up-regulated; black colour, represent no expression differences compared to dry seeds. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds

imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

5.3.4 Changes in gene expression between un-germinated and germinated seeds

The hierarchical clustering of genes with expression differences greater than 2.5 fold between germinated and un-germinated seeds is shown in Figure 5.4. There were clear differences between germinated and un-germinated seeds for all treatments. As expected, there were a large increase in both the number and degree of expression of genes during germination. The greater differences occurred in seeds imbibed in either the saline solution or ABA (Figures 5.4 and 5.5). For seeds imbibed in GA_{4+7}, 262 genes were differentially expressed between germinated and un-germinated seeds, 398 for seeds imbibed in water, 723 for seeds imbibed in the saline solution and 1008 for seeds imbibed in ABA (Figure 5.5). Major genes differentially expressed were selected and grouped into four groups as displayed in Tables 5.2, 5.3, 5.4 and 5.5. These four groups were LEA and stress-related, hormone-related, hydrolase-related and specific seed germination-related genes.

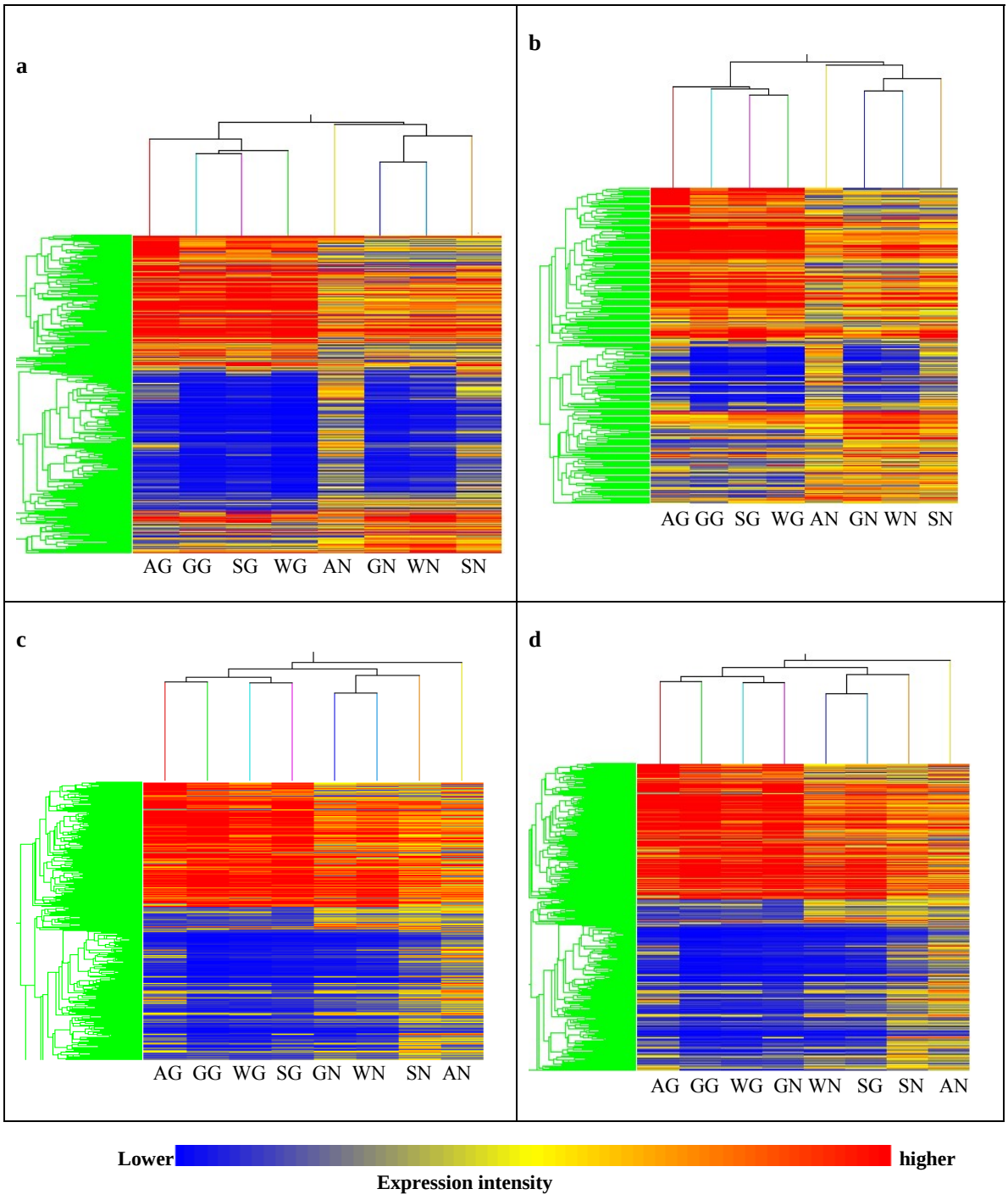


Figure 5.4. Hierarchical clustering of genes with expression differences greater than 2.5 fold between un germinated and geminated seeds imbibed in either water, GA_{4+7}, a saline solution or ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA. **a**. Water; **b**. GA_{4+7}; **c**. a saline solution; **d**. ABA.

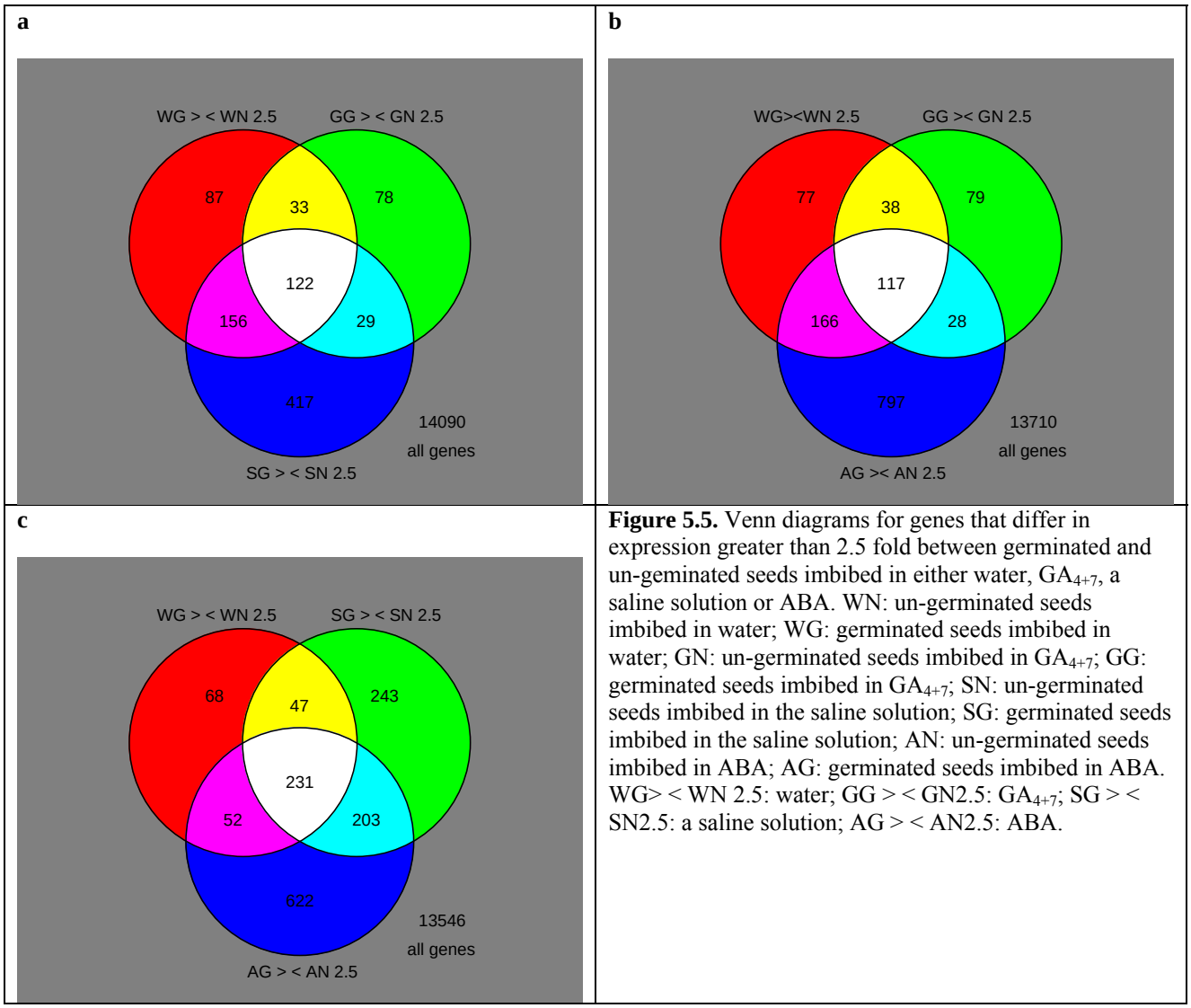


Figure 5.5. Venn diagrams for genes that differ in expression greater than 2.5 fold between germinated and un-geminated seeds imbibed in either water, GA_{4+7}, a saline solution or ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA. WG> < WN 2.5: water; GG > < GN2.5: GA_{4+7}; SG > < SN2.5: a saline solution; AG > < AN2.5: ABA.

Table 5.2. Putative genes with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in water.

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN10961	LEA group 1 domain-containing protein similarity to DS11	0.011
	BN16767	LEA protein, putative similar to Lea14-A {*Gossypium hirsutum*}	0.004
	BN19370	glycine-rich protein / late embryogenesis abundant protein (M17)	0.017
	BN19387	late embryogenesis abundant protein (M10) / LEA protein M10	0.004
	BN20144	putative / LEA protein, similar to LEA 76 {*Brassica napus*}	0.010
	BN20349	LEA domain-containing, low similarity to Desiccation-related protein	0.002
	BN20598	LEA domain-containing protein, low similarity to D-29	0.007
	BN20688	LEA protein, nearly identical to LEA protein in group 3(ECP63)	0.019
	BN20893	LEA domain-containing, low similar to 51 kDa seed maturation protein	0.016
	BN24859	LEA group 1 domain-containing protein	0.013
	BN25166	LEA domain-containing, low similarity to embryogenic abundant gene	0.004

	BN26527	putative / LEA protein, putative similar to LEA protein in group 3	0.006
	BN10431	dehydrin (RAB18), nearly identical to Dehydrin Rab18	0.004
	BN11240	identical to dehydration-induced protein (ERD15)	0.205
	BN11528	senescence/dehydration-associated protein-related (ERD7)	0.122
	BN20125	dehydrin family protein	0.007
	BN23186	similar to drought-induced mRNA	0.364
	BN12067	putative similar to temperature stress-induced lipocalin	0.064
	BN12218	ABA-responsive protein-related, similar to cold-induced protein kin1	0.063
	BN15778	dormancy/auxin associated family protein	0.012
	BN19033	phosphatidylethanolamine-binding, similar to cold-regulated protein	0.011
	BN20755	ABA-responsive protein (HVA22b), identical to AtHVA22b	0.019
Group	BN24919	stress-responsive protein,related to Desiccation-responsive protein 29B	0.005
1	BN12279	plant defensin-fusion protein, putative (PDF1.2c)	0.015
	BN13968	plant defensin protein, putative (PDF1.2a)	0.044
	BN14537	plant defensin-fusion protein, putative (PDF1.2b)	0.040
	BN19962	plant defensin-fusion protein, putative (PDF1.4)	0.010
	BN14817	17.6 kDa class II heat shock protein (HSP17.6-CII)	0.007
	BN15189	DNAJ heat shock N-terminal domain-containing, similarity to HSP40	0.209
	BN25006	identical to heat shock protein 101 (HSP101)	0.011
Group	BN15701	GDSL-motif lipase/hydrolase protein, similar to early nodulin ENOD8	0.395
2	BN26772	glycosyl hydrolase family 1 protein contains	0.165
Group	BN20519	gibberellin-regulated protein 3 precursor (GASA3)	0.023
3	BN20885	gibberellin-regulated protein 2 precursor (GASA2)	0.011
	BN11682	Similar to cytochrome P450 from [Catharanthus roseus]	0.345
	BN12371	protein phosphatase 2C, putative / PP2C from [*Lotus japonicus*]	0.165
	BN15670	putative similar to cytochrome P450 from [*Catharanthus roseus*]	0.180
Group	BN16242	protein phosphatase 2C, putative / PP2C from [*Arabidopsis thaliana*]	0.204
4	BN18693	bZIP transcription factor (OBF4), identical to ocs-element binding factor	0.254
	BN24369	putative similar to Cytochrome P450 91A1	0.019
	BN25156	similar to ABA-responsive element binding protein 1 (AREB1) bZIP	0.121
	BN26343	protein phosphatase 2C, putative / PP2C EC 3.1.3.16	0.046
	BN10327	LEA family protein, similar to ethylene-responsive LEA-like protein	0.120
	BN10217	identical to Dehydrin ERD14 {Arabidopsis thaliana}	13.467
Group	BN11426	dehydrin family protein	3.622
5	BN11835	stress-induced protein (KIN2) / cold-responsive protein (COR6.6)	8.426
	BN12729	identical to Dehydration-responsive protein RD22 precursor	2.999
	BN15343	cold-acclimation protein, putative (FL3-5A3)	6.167
	BN14158	related to Wound-induced protein 1 {*Solanum tuberosum*}	3.068
	BN10737	glycosyl hydrolase family 1 / beta-glucosidase, putative (BG1)	3.526
	BN13810	glycosyl hydrolase family 1 protein	6.896
	BN15161	glycosyl hydrolase family 3 protein	23.897
	BN16742	glycosyl hydrolase family 1 protein	15.276
Group	BN16895	beta-galactosidase, putative / lactase	9.282
6	BN19666	glycosyl hydrolase family 17, similar to elicitor inducible chitinase	3.468
	BN19799	alpha-xylosidase (XYL1) identical to alpha-xylosidase precursor	17.276
	BN20118	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL1	50.419
	BN20681	expansin family protein (EXPL2)	4.496
	BN21244	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	3.234
	BN21616	beta-galactosidase, putative / lactase [*Lycopersicon esculentum*]	15.223
	BN22148	glycosyl hydrolase family 17, similar to endo-1,3-beta-glucosidase	7.024
	BN22579	GDSL-motif /hydrolase family, low similarity to family II lipaseEXL1	5.476

	BN23747	proline-rich extensin-like family protein	15.594
Group 6	BN23885	glycosyl hydrolase family 35 protein, similar to beta-galactosidases	5.895
	BN26760	chitinase, putative similar to basic endochitinase CHB4 precursor	5.287
	BN27626	glycosyl hydrolase family 1 protein / beta-glucosidase, putative (BG1)	7.333
Group 7	BN11763	Gibberellin-regulated protein 4 precursor GASA4	10.101
	BN22163	similar to auxin-responsive GH3 product [*Glycine max*]	3.618
	BN24370	2OG-Fe(II) oxygenase family, low similarity to gibberellin 20-oxidase	28.635
Group 8	BN10224	plasma membrane intrinsic protein 2B (PIP2B) / aquaporin PIP2.2	4.929
	BN10387	plasma membrane intrinsic protein 1B (PIP1B) / aquaporin PIP1.2	5.602
	BN17421	cytochrome P450 71B28, putative (CYP71B28)	3.912
	BN22218	identical to cDNA putative myb transcription factor (At5g49330)	4.180
	BN22648	cell division control protein	2.509
	BN24616	cytochrome P450, putative	2.796
	BN24871	bZIP transcription factor family protein	4.686
	BN26349	cytochrome P450 71B22, putative (CYP71B22)	2.679

Four specific groups of genes, with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in water. Group 1-4: down regulated in germinated seeds; Group 5-8: up-regulated in germinated seeds.

Group1: LEA and stress related genes; Group 2: hydrolase related genes; Group 3: Hormone related genes; Group 4: specific seed germination related genes.

Group 5: LEA genes, stress related; Group 6: hydrolase related; Group 7: Hormone related; Group 8: specific seed germination related genes. Relative ratio= samples/control (dry seeds).

Table 5.3. Putative genes with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in GA_{4+7}.

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN10961	LEA group 1 domain-containing protein similarity to DS11	0.022
	BN20144	putative / LEA protein, similar to LEA 76 {*Brassica napus*}	0.021
	BN20349	LEA domain-containing, low similarity to Desiccation-related protein	0.006
	BN20688	LEA protein, nearly identical to LEA protein in group 3(ECP63)	0.032
	BN20893	LEA domain-contain, low similarity to 51 kDa seed maturation protein	0.030
	BN24859	LEA group 1 domain-containing protein	0.034
	BN25166	LEA domain-contain, low similarity to embryogenic abundant protein	0.009
	BN10431	dehydrin (RAB18), nearly identical to Dehydrin Rab18	0.009
	BN11240	identical to dehydration-induced protein ERD15	0.257
	BN11528	senescence/dehydration-associated protein-related (ERD7)	0.113
	BN12218	ABA-responsive protein-related, similar to ABA-inducible protein	0.143
	BN20755	ABA-responsive protein (HVA22b), identical to AtHVA22b	0.038
	BN13114	31.2 kDa small heat shock family protein(hsp20)	0.248
	BN17923	putative similar to wound induced protein [*Lycopersicon esculentum*]	0.264
	BN11007	related to wound-responsive protein-tomato (fragment)	0.048
Group 2	BN12525	glycosyl hydrolase family 1 protein	0.352
Group 3	BN20885	Gibberellin-regulated protein 2 precursor (GASA2)	0.026
Group 4	BN11682	similar to cytochrome P450 from [*Catharanthus roseus*]	0.254
	BN15670	putative similar cytochrome P450 to from [*Catharanthus roseus*]	0.171
	BN16242	protein phosphatase 2C, putative / PP2C from [*Arabidopsis thaliana*]	0.274

Group 4	BN25156	similar to ABA-responsive element binding protein 1 (AREB1) (bZip)	0.114
	BN26343	protein phosphatase 2C, putative / PP2C (EC 3.1.3.16)	0.044
Group 5	BN10327	LEA family protein, similar to ethylene-responsive LEA-like protein	3.015
	BN10217	dehydrin (ERD14) {Arabidopsis thaliana}	8.683
	BN12729	identical to Dehydration-responsive protein RD22 precursor	3.376
	BN15343	cold-acclimation protein, putative (FL3-5A3)	3.328
	BN18389	Hydrophobic RCI2B, Low temperature and salt responsive LTI6B	3.124
	BN14158	related to Wound-induced protein 1 {*Solanum tuberosum*}	2.616
Group 6	BN15161	glycosyl hydrolase family 3 protein	13.482
	BN16742	glycosyl hydrolase family 1 protein	8.789
	BN20118	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL1	23.939
	BN21244	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	7.426
	BN22148	glycosyl hydrolase family 17, similar to endo-1,3-beta-glucosidase	8.235
	BN23747	proline-rich extensin-like family protein	23.048
	BN23885	glycosyl hydrolase family 35 protein, similar to beta-galactosidase	5.126
	BN24266	similar to basic endochitinase CHB4 precursor from [Brassica napus]	3.816
	BN24511	GDSL-motif lipase/hydrolase family protein similar to Enod8.1	3.030
	BN25419	glycoside hydrolase family 28/polygalacturonase (pectinase) family	15.087
	BN26760	chitinase, putative similar to basic endochitinase CHB4 precursor	3.283
	BN27626	glycosyl hydrolase family 1 protein / beta-glucosidase, putative (BG1)	4.546
Group 7	BN11763	Gibberellin-regulated protein 4 precursor GASA4	6.822
	BN22163	similar to auxin-responsive GH3 product [*Glycine max*]	2.501
	BN22657	auxin-responsive protein / indoleacetic acid-induced protein 4 (IAA4)	17.791
	BN24370	2OG-Fe(II) oxygenase family, low similarity to gibberellin 20-oxidase	17.707
Group 8	BN10224	plasma membrane intrinsic protein 2B (PIP2B) / aquaporin PIP2.2	3.388
	BN10387	plasma membrane intrinsic protein 1B (PIP1B) / aquaporin PIP1.2	3.198
	BN22218	identical to cDNA putative myb transcription factor (At5g49330)	2.767
	BN22648	cell division control protein	3.473
	BN24616	cytochrome P450, putative	2.542
	BN24871	bZIP transcription factor family protein	3.507
	BN26349	cytochrome P450 71B22, putative (CYP71B22)	3.057

Four specific groups of genes as described in table 5.2 with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in GA_{4+7}.

Table 5.4. Putative genes with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in a saline solution.

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN20598	LEA domain-containing protein, low similarity to D-29	0.034
	BN24859	LEA group 1 domain-containing protein	0.200
	BN25166	LEA domain-containing, low similarity to embryogenic abundant gene	0.036
	BN26527	putative /LEA protein, putative similar to LEA protein in group 3	0.050
	BN10431	dehydrin (RAB18), nearly identical to Dehydrin Rab18	0.016
	BN16472	early-responsive to dehydration stress protein (ERD4)	0.128
	BN20009	similarity to drought-induced mRNA, Di19 [*Arabidopsis thaliana*]	0.299
	BN24919	stress-responsive protein-related to Desiccation-responsive protein 29B	0.033
	BN26935	DRE-binding protein (DREB2B), identical to DREB2B	0.049
	BN20902	25.3 kDa small heat shock protein, chloroplast precursor (HSP25.3-P)	0.040
	BN17923	putative similar to wound induced protein [*Lycopersicon esculentum*]	0.299

Group 1	BN19511	universal stress protein (USP) family protein similar to ER6 protein	0.024
	BN19962	plant defensin-fusion protein, putative (PDF1.4)	0.030
	BN24959	universal stress protein (USP) family protein	0.043
Group 2	BN15448	GDSL-motif /hydrolase family, similar to myrosinase-associated protein	0.239
	BN18205	glycosyl hydrolase family 14 protein, similar to beta-amylase enzyme	0.309
	BN18758	hydrolase, low similarity to monoglyceride lipase from [*Homo sapiens*]	0.181
	BN24651	hydrolase, alpha/beta fold family protein	0.380
Group 3	BN12888	zeaxanthin epoxidase (ZEP) (ABA1), identical to AtABA1	0.229
	BN20519	Gibberellin-regulated protein 3 precursor (GASA3)	0.070
	BN20885	Gibberellin-regulated protein 2 precursor (GASA2)	0.042
	BN25600	auxin-responsive protein, putative auxin-inducible SAUR gene	0.389
	BN27085	identical to ethylene receptor 1 (ETR1)	0.298
Group 4	BN10846	AP2 domain-containing transcription factor, putative EREBP-3 homolog	0.080
	BN12371	protein phosphatase 2C, putative / PP2C [*Lotus japonicus*]	0.205
	BN14671	similarity to WRKY-type DNA-binding transcription factor protein	0.063
	BN16242	protein phosphatase 2C, putative / PP2C from [*Arabidopsis thaliana*]	0.246
	BN16341	putative similar to AP2 domain containing protein RAP2.1	0.047
	BN18693	bZIP transcription factor (OBF4), identical to ocs-element binding factor	0.341
	BN18945	golden2-like transcription factor (GLK1)	0.066
	BN19210	bZIP family transcription factor, similar to bZIP transcription factor	0.061
	BN21817	myb family transcription factor	0.151
	BN22460	myb family transcription factor	0.246
	BN24618	identical to cytochrome P450 71B20	0.282
	BN25156	similar to ABA-responsive element binding protein 1 (AREB1)	0.239
	BN25763	protein phosphatase 2C / PP2C, abscisic acid-insensitive 2 (ABI2)	0.311
	BN26682	similar to Cytochrome P450 90C1 (ROTUNDIFOLIA3)	0.099
Group 5	BN10195	identical to dehydrin COR47 (Cold-induced COR47 protein)	3.165
	BN10217	identical to Dehydrin ERD14 {Arabidopsis thaliana}	14.497
	BN10905	similar to early-responsive to dehydration stress ERD3 protein	10.184
	BN10936	putative strong similarity to Heat shock protein 81-2 (HSP81-2)	3.081
	BN11835	stress-induced protein (KIN2) / cold-responsive protein (COR6.6)	6.796
	BN11999	similar to DnaJ homolog subfamily B member 11 precursor	3.521
	BN12729	identical to Dehydration-responsive protein RD22 precursor	7.184
	BN13096	putative strong similarity to Heat shock protein 81-2 (HSP81-2)	3.227
	BN13804	similar to early-responsive to dehydration stress ERD3 protein	3.518
	BN14158	related to Wound-induced protein 1 {Solanum tuberosum}	3.826
	BN17226	plant defensin-fusion protein, putative (PDF2.1)	6.485
	BN18389	Hydrophobic RCI2B, Low temperature and salt responsive LTI6B	3.908
	BN21684	similar to early-responsive to dehydration stress ERD3 protein	3.532
Group 6	BN13433	chitinase-like protein 1 (CTL1), similar to class I chitinase	5.355
	BN13445	glycosyl hydrolase family 17, similar to beta-1,3-glucanase precursor	7.816
	BN16604	GDSL-motif lipase/hydrolase family, similar to lipase	21.196
	BN16784	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	17.840
	BN16811	GDSL-motif lipase/hydrolase family protein	16.459
	BN16895	putative similar to beta-galactosidase precursor	26.368
	BN20118	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL1	42.321
	BN22148	glycosyl hydrolase family 17, similar to endo-1,3-beta-glucosidase,	17.478
	BN26760	chitinase, putative similar to basic endochitinase CHB4 precursor	19.790

	BN11763	Gibberellin-regulated protein 4 precursor GASA4	11.574
	BN12316	1-aminocyclopropane-1-carboxylate synthase / ACC synthase	2.744
	BN14737	auxin-responsive / indoleacetic acid-induced protein 16 (IAA16)	8.196
	BN19321	similarity to amino-cyclopropane-carboxylic acid oxidase (ACC ox2)	4.463
Group	BN22163	similar to auxin-responsive GH3 product [Glycine max]	3.067
7	BN22657	auxin-responsive protein / indoleacetic acid-induced protein 4 (IAA4)	29.344
	BN22844	similar to Gibberellin-regulated protein 1 precursor	10.779
	BN24370	2OG-Fe(II) oxygenase family, low similarity to gibberellin 20-oxidase	36.637
	BN10224	plasma membrane intrinsic protein 2B (PIP2B) / aquaporin PIP2.2	7.403
	BN10387	plasma membrane intrinsic protein 1B (PIP1B) / aquaporin PIP1.2	4.913
	BN11437	plasma membrane intrinsic protein 2C (PIP2C) / aquaporin PIP2.3	6.369
Group	BN15095	plasma membrane intrinsic protein 2A (PIP2A) / aquaporin PIP2.1	2.702
8	BN14194	myb family transcription factor	4.089
	BN15837	myb family transcription factor	3.226
	BN22648	cell division control protein	2.360
	BN23743	identical to b-Zip DNA binding protein	4.624
	BN24871	bZIP transcription factor family protein	3.671
	BN27674	protein phosphatase 2C/PP2C, abscisic acid-insensitive 1 (ABI1)	3.223

Four specific groups of genes as described in table 5.2 with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in the saline solution.

Table 5.5. Putative genes with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in the ABA.

Groups	Clone ID	Gene Names	Relative Ratio
	BN10961	LEA group 1 domain-containing protein low similarity toDS11	0.308
	BN16767	LEA protein, putative similar to Lea14-A {*Gossypium hirsutum*}	0.043
	BN20144	putative / LEA protein, similar to LEA 76 {*Brassica napus*}	0.356
	BN24859	LEA group 1 domain-containing protein	0.375
	BN24993	expressed protein very low similarity to LEA protein [*Cicer arietinum*]	0.076
	BN25011	LEA group 1 domain-containing protein	0.244
	BN25166	LEA domain-containing, low similarity to embryogenic abundant gene	0.090
Group	BN20009	similarity to drought-induced mRNA, Di19 [*Arabidopsis thaliana*]	0.263
1	BN20125	dehydrin family protein	0.211
	BN20755	ABA-responsive protein (HVA22b), identical to AtHVA22b	0.366
	BN26935	DRE-binding protein (DREB2B), identical to DREB2B	0.054
	BN12619	universal stress protein (USP) family protein	0.336
	BN17568	USP family protein,similar to ethylene-responsive ER6 protein	0.103
	BN17923	putative similar to wound induced protein [*Lycopersicon esculentum*]	0.308
	BN19511	universal stress protein (USP) family protein similar to ER6 protein	0.047
	BN19962	plant defensin-fusion protein, putative (PDF1.4)	0.034
	BN24959	universal stress protein (USP) family protein	0.088
	BN10667	DNAJ heat shock N-terminal domain-containing proteins	0.086
	BN11376	putative similar to heat shock protein hsp70 from [*Pisum sativum*]	0.034
	BN14817	17.6 kDa class II heat shock protein (HSP17.6-CII)	0.034
	BN15062	DNAJ heat shock N-terminal domain-containing protein	0.308
	BN15189	DNAJ heat shock N-terminal domain-containing, similarity to HSP40	0.241
	BN15627	DNAJ heat shock family (Heat shock 40 kDa protein 1 homolog)	0.019
	BN17514	identical to heat shock protein 70	0.072
	BN19713	similar to DNAJ heat shock N-terminal domain-containing CAJ1 protein	0.338
	BN23457	cold-shock DNA-binding family protein	0.380
	BN25006	identical to heat shock protein 101 (HSP101)	0.038

	BN27679	similarity to DNAJ heat shock N-terminal domain-containing protein J2	0.372
Group 2	BN15448	GDSL-motif /hydrolase family, similar to myrosinase-associated protein	0.392
	BN24587	glycosyl hydrolase family 17, similar to inducible chitinase Nt-SubE76	0.228
	BN27593	glycosyl hydrolase family 1 protein	0.112
Group 3	BN12888	zeaxanthin epoxidase (ZEP) (ABA1), identical to AtABA1	0.392
	BN15727	identical to Ethylene responsive element binding factor 2 (AtERF2)	0.335
	BN20519	Gibberellin-regulated protein 3 precursor (GASA3)	0.163
	BN20885	Gibberellin-regulated protein 2 precursor (GASA2)	0.151
	BN22832	auxin-responsive family, similar to auxin-induced protein AIR12	0.325
	BN27085	identical to ethylene receptor 1 (ETR1)	0.290
Group 4	BN10178	similar to AP2 domain-containing protein RAP2.3 (RAP2.3)	0.154
	BN12371	protein phosphatase 2C, putative / PP2C [Lotus japonicus]	0.268
	BN14671	similarity to WRKY-type DNA-binding transcription factor protein	0.070
	BN15670	putative similar to cytochrome P450	0.249
	BN16341	putative similar to AP2 domain containing protein RAP2.1	0.077
	BN17285	similarity to WRKY-type DNA-binding transcription factor protein	0.073
	BN18567	Identical to Cytochrome P450 71B23	0.363
	BN18693	bZIP transcription factor (OBF4), identical to ocs-element binding factor	0.220
	BN21037	putative similar to cytochrome P450 89A2	0.153
	BN21176	similar to AP2 domain-containing transcription factor TINY	0.224
	BN21817	myb family transcription factor	0.214
	BN22460	myb family transcription factor	0.269
	BN24369	putative similar to Cytochrome P450 91A1 [*Arabidopsis thaliana*]	0.086
	BN24555	identical to Cytochrome P450 71B5	0.110
	BN24618	identical to cytochrome P450 71B20	0.212
	BN26343	protein phosphatase 2C, putative / PP2C (EC 3.1.3.16)	0.091
	BN26536	germin-like protein, putative similar to germin -like protein GLP6	0.116
	BN26682	similar to Cytochrome P450 90C1 (ROTUNDIFOLIA3)	0.349
Group 5	BN10327	LEA family protein, similar to ethylene-responsive LEA-like protein	4.580
	BN10195	identical to dehydrin COR47 (Cold-induced COR47 protein)	12.173
	BN10217	identical to Dehydrin (ERD14) {Arabidopsis thaliana}	21.644
	BN10637	universal stress protein (USP) ,similar to early nodulin ENOD18	16.395
	BN10936	identical to dehydrin ERD10, Low-temperature-induced protein LTI45	14.411
	BN11426	dehydrin family protein	7.050
	BN11835	stress-induced protein (KIN2) / cold-responsive protein (COR6.6)	35.472
	BN12729	identical to Dehydration-responsive protein RD22 precursor	4.126
	BN13804	similar to early-responsive to dehydration stress ERD3 protein	3.139
	BN13942	similar to early-responsive to dehydration stress protein (ERD3)	2.928
	BN13977	stress-inducible protein, similar to sti (stress inducible protein)	3.199
	BN15067	universal stress protein (USP) family protein similar to ER6	23.802
	BN15343	cold-acclimation protein, putative (FL3-5A3)	21.380
	BN17214	LTI65 / desiccation-responsive protein 29B (RD29B)	3.829
	BN18389	Hydrophobic RCI2B, Low temperature and salt responsive LTI6B	10.714
	BN21684	similar to early-responsive to dehydration stress ERD3 protein	4.581
Group 6	BN10737	glycosyl hydrolase family 1 / beta-glucosidase, putative (BG1)	13.761
	BN13433	chitinase-like protein 1 (CTL1), similar to class I chitinase	8.790
	BN15161	glycosyl hydrolase family 3 protein	18.719
	BN15790	epoxide hydrolase, similar to epoxide hydrolase (*Solanum tuberosum*)	4.008
	BN16604	GDSL-motif lipase/hydrolase family protein, similar to lipase	28.220
	BN16742	glycosyl hydrolase family 1 protein	18.999
	BN16784	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	15.726

	BN16811	GDSL-motif lipase/hydrolase family protein	22.501
	BN16883	GDSL-motif lipase, putative similar to lipase [*Arabidopsis thaliana*]	3.127
	BN18214	glycosyl hydrolase family 3 protein	15.243
	BN18721	GDSL-motif lipase/hydrolase family, similar to early nodulin ENOD8	7.193
	BN19126	Cell wall invertase identical to beta-fructofuranosidase	12.483
	BN19596	glycoside hydrolase28/ polygalacturonase (pectinase) family protein	12.487
	BN19799	alpha-xylosidase (XYL1) identical to alpha-xylosidase precursor	34.445
	BN19871	glycosyl hydrolase family 1 protein contains	25.697
Group	BN20118	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL1	55.563
6	BN21355	putative similar to endo-beta-1,4-glucanase from [*Fragaria x ananassa*]	7.318
	BN21616	putative similar to beta-galactosidase [*Lycopersicon esculentum*]	41.351
	BN22754	GDSL-motif /hydrolase family protein low similarity to APG precursor	5.658
	BN23747	proline-rich extensin-like protein	45.568
	BN24266	similar to basic endochitinase CHB4 precursor from *[Brassica napus*]	4.468
	BN24468	glycosyl hydrolase family 1 protein	3.614
	BN25325	putative similar to beta-galactosidase precursor	5.422
	BN26760	chitinase, putative similar to basic endochitinase CHB4 precursor	10.841
	BN27052	alpha-glucosidase 1 (AGLU1), identical to alpha-glucosidase 1	18.333
	BN27626	glycosyl hydrolase family 1 protein / beta-glucosidase, putative (BG1)	33.496
	BN10799	calcium-binding RD20 protein induced by ABA during dehydration	7.552
	BN11763	Gibberellin-regulated protein 4 precursor GASA4	7.579
	BN12534	auxin-responsive protein / indoleacetic acid-induced protein 2 (IAA2)	4.048
	BN13131	similar to auxin-responsive GH3 product	16.249
Group	BN14737	auxin-responsive / indoleacetic acid-induced protein 16 (IAA16)	13.809
7	BN16638	nearly identical to ACC oxidase (ACC ox1) from [*Brassica oleracea*]	36.953
	BN16876	putative (PIN3), similar to auxin transport protein	7.011
	BN19321	similarity to amino-cyclopropane-carboxylic acid oxidase (ACC ox2)	20.294
	BN22163	similar to auxin-responsive GH3 product [*Glycine max*]	3.757
	BN22844	Gibberellin-regulated protein 1 precursor GASA 1	19.070
	BN23039	identical to ethylene response sensor (ERS)	16.457
	BN23715	similar to ACC oxidase [Sorghum bicolor]	32.570
	BN24370	2OG-Fe(II) oxygenase family, low similarity to gibberellin 20-oxidase	26.135
	BN25755	transcriptional factor B3 family protein / auxin-responsive factor	2.686
	BN10224	plasma membrane intrinsic protein 2B (PIP2B) / aquaporin PIP2.2	4.663
	BN10387	plasma membrane intrinsic protein 1B (PIP1B) / aquaporin PIP1.2	5.831
	BN11437	plasma membrane intrinsic protein 2C (PIP2C) / aquaporin PIP2.3	5.300
	BN15095	plasma membrane intrinsic protein 2A (PIP2A) / aquaporin PIP2.1	3.659
	BN17472	aquaporin, putative similar to plasma membrane aquaporin 2b	27.049
	BN20259	plasma membrane intrinsic protein 1C (PIP1C) / aquaporin PIP1.3	7.027
Group	BN11644	AP2 domain-containing transcription factor RVA2	7.854
8	BN11645	AP2 domain-containing transcription factor,	5.965
	BN12117	GER3, identical to germin-like protein subfamily 3 member 3	4.450
	BN12157	AP2 domain-containing transcription factor, putative similar to RAV1	3.953
	BN12746	raffinose synthase / seed imbibition protein, putative (din10)	14.525
	BN13436	luminal binding protein 1 (BiP-1) (BP1)	13.644
	BN22351	bZIP protein	2.758
	BN22648	cell division control protein	3.155
	BN23743	identical to bZIPDNA binding transcription factor family protein	3.263
	BN24406	similar to myb-related transcription activator	4.384
	BN24871	bZIP transcription factor family protein	4.303
	BN27674	protein phosphatase 2C/ PP2C, abscisic acid-insensitive 1 (ABI1)	2.816

Four specific groups of genes as described in table 5.2 with expression differences greater than 2.5 fold between germinated and un-germinated seeds imbibed in ABA.

5.3.5 A Comparison of gene expression in un-germinated seeds imbibed in either water, GA_{4+7}, the saline solution or ABA

Compared to water imbibed un-germinated seeds, 49 genes were differentially expressed in un-germinated seeds imbibed in GA_{4+7}, 582 for un-germinated seeds imbibed in saline solution and 1320 for un-germinated seeds imbibed in ABA (Figure 5.6). There were less differences in these gene expression for the treatments in germinated seeds (Figure 5.7). There were smaller difference between water and GA_{4+7} treated seeds compared to seeds imbibed in the saline solution or ABA (Figure 5.7). Four groups of genes as LEA and stress-related, hormone related, hydrolase-related and specific seed germination-related genes were presented in Tables 5.6, 5.7, 5.8 and 5.9.

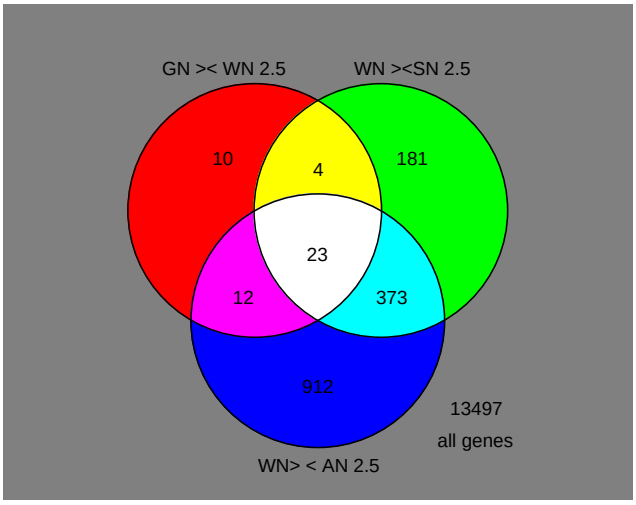


Figure 5.6. Venn diagrams for genes that differ in expression greater than 2.5 fold between un-germinated seeds imbibed in water and the treatments exerted. GN > < WN: seeds imbibed in GA_{4+7}; WN > < SN: seeds imbibed in a saline solution; WN > < AN: seeds imbibe in ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

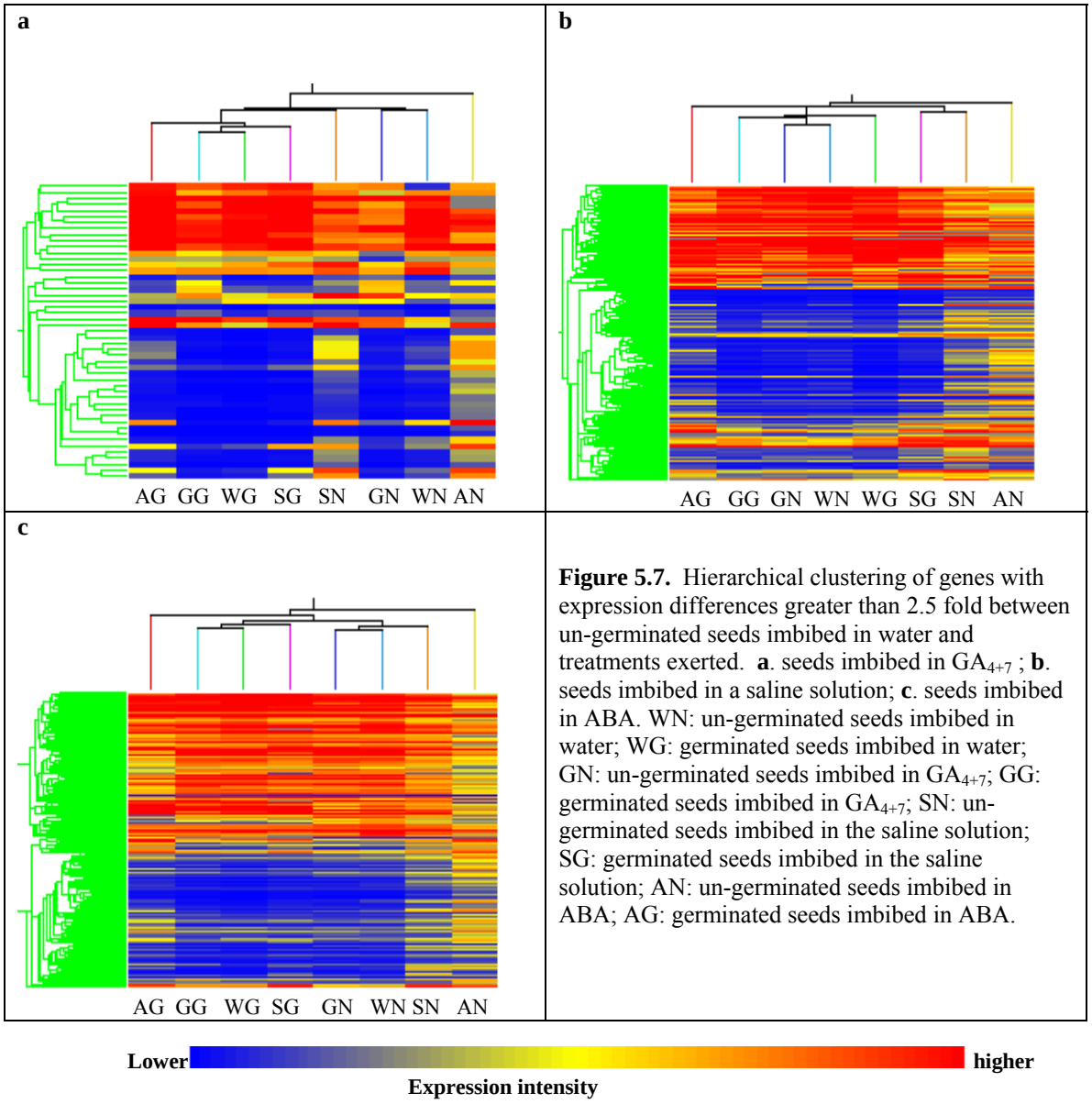


Figure 5.7. Hierarchical clustering of genes with expression differences greater than 2.5 fold between un-germinated seeds imbibed in water and treatments exerted. **a**. seeds imbibed in GA_{4+7} ; **b**. seeds imbibed in a saline solution; **c**. seeds imbibed in ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

Table 5.6. Putative genes with expression differences greater than 2.5 fold between un-germinated seeds imbibed in water and GA_{4+7}.

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN19370	identical to late-embryogenesis abundant M17 protein	0.099
	BN19387	late embryogenesis abundant protein (M10) / LEA protein M10	0.061
	BN10459	related to wound inducive gene [Nicotiana tabacum]	0.358
	BN11063	DNAJ heat shock protein, similar to heat shock 40 kDa protein 1	0.381
	BN11528	senescence/dehydration-associated protein-related (ERD7)	0.300
	BN11573	identical to dnaJ heat shock protein J11	0.393
	BN12279	plant defensin-fusion protein, putative (PDF1.2c)	0.151
	BN14537	plant defensin-fusion protein, putative (PDF1.2b)	0.124

Group 1	BN15778	dormancy/auxin associated family protein	0.074
	BN17428	plant defensin-fusion protein, putative (PDF2.2)	0.361
	BN18222	cold-shock DNA-binding family protein	0.300
	BN19341	wound-responsive protein-related, weak similarity to KED	0.395
	BN19962	plant defensin-fusion protein, putative (PDF1.4)	0.056
	BN20738	identical to 18.2 kDa class I heat shock protein (HSP 18.2)	0.028
	BN21069	DNAJ heat shock N-terminal domain-contain, low similarity to AHM1	0.381
	BN22615	heat shock protein 100, putative / HSP100,	0.370
	BN23509	universal stress protein , low similarity to early nodulin ENOD18	0.356
Group 2	BN19506	xyloglucan:xyloglucosyl transferase / xyloglucan endotransglycosylase	0.390
	BN20407	glycosyl hydrolase family 38 protein similar to alpha-mannosidase	0.371
	BN26721	glycoside hydrolase family 2 protein low similarity to mannosidase	0.384
	BN26772	Similar to glycosyl hydrolase family 1, Cyanogenic Beta-Glucosidase	0.124
Group 3	N/A	N/A	N/A
Group 4	BN12189	protein phosphatase 2C family protein / PP2C family protein	0.393
	BN15063	identical to Arabidopsis germin-like protein subfamily 2 member 1	0.345
	BN15915	myb family transcription factor (GLK2)	0.351
	BN19766	identical to 12S seed storage protein (CRA1)	0.010
	BN22460	myb transcription factor contains myb-like DNA-binding domain	0.352
	BN25156	similar to ABA-responsive element binding protein 1 (AREB1) bZIP	.364 0.330
Group 5	BN12731	dehydration-responsive protein (RD22)	2.815
Group 6	BN14129	Expansin, putative (EXP5), identical to expansin At-EXP5	7.647
	BN16895	beta-galactosidase, putative / lactase	12.139
	BN19799	alpha-xylosidase (XYL1) identical to alpha-xylosidase precursor	8.570
	BN20681	expansin family protein (EXPL2)	2.929
	BN23761	expansin, putative (EXP4) similar to alpha-expansin 6 precursor	6.698
Group 7	N/A	N/A	N/A
Group 8	BN12157	AP2 domain-containing transcription factor, putative similar to RAV1	2.583
	BN15911	myb family transcription factor (MYB30) identical to myb-like protein	10.287
	BN21442	myb family transcription factor	3.575
	BN22991	RNA recognition motif (RRM)-containing protein	11.339
	BN25946	myb family transcription factor (KAN1)	3.726

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down regulated; Group 5-8: up-regulated.

Table 5.7. Putative genes depressed by both a saline solution and ABA in un-germinated seeds as compared to water imbibed un-germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN10961	LEA group 1 domain-containing protein similarity to DS11	0.144
	BN16767	LEA protein, putative similar to Lea14-A {*Gossypium hirsutum*}	0.024
	BN19387	late embryogenesis abundant protein (M10) / LEA protein M10	0.061
	BN20144	putative / LEA protein, similar to LEA 76 {*Brassica napus*}	0.119

	BN20349	LEA domain-containing, low similarity to desiccation-related protein	0.045
	BN20598	LEA domain-containing protein, low similarity to D-29	0.031
	BN20688	LEA protein, nearly identical to LEA protein in group 3(ECP63)	0.112
	BN20893	LEA domain-containing, low similar to 51 kDa seed maturation protein	0.206
	BN24859	LEA group 1 domain-containing protein	0.12
	BN25011	LEA group 1 domain-containing protein	0.067
	BN26527	putative / LEA protein, putative similar to LEA protein in group 3	0.06
	BN10431	dehydrin (RAB18), nearly identical to Dehydrin Rab18	0.033
	BN10667	DNAJ heat shock N-terminal domain-containing protein	0.027
	BN11007	related to wound-induced protein - tomato (fragment)	0.221
	BN11376	putative similar to heat shock protein hsp70 from [*Pisum sativum*]	0.018
	BN11635	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	0.027
	BN12279	plant defensin-fusion protein, putative PDF1.2c	0.151
Group	BN12981	universal stress protein (USP) family protein similar to ER6 protein	0.043
1	BN13289	nearly identical to stress enhanced protein 2 (SEP2)	0.172
	BN13427	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	0.107
	BN13968	plant defensin protein, putative (PDF1.2a)	0.116
	BN14450	identical to heat shock transcription factor 4 (HSF4)	0.255
	BN14537	plant defensin-fusion protein, putative (PDF1.2b)	0.124
	BN14817	identical to 17.6 kDa class II heat shock protein	0.019
	BN14863	identical to dormancy-associated protein DRM1	0.385
	BN15452	identical to 17.4 kDa class I heat shock protein (HSP17.4-CI)	0.011
	BN15627	DNAJ heat shock family (Heat shock 40 kDa protein 1 homolog)	0.01
	BN15648	identical to 17.6 kDa class I heat shock protein (HSP 17.6)	0.017
	BN15854	plant defensin-fusion protein, putative (PDF2.3)	0.271
	BN16309	weak similarity to HSF 1 (Heat shock transcription factor 1) (HSTF 1)	0.374
	BN16590	similar to early-responsive to dehydration stress ERD3 protein	0.362
	BN17057	similar to 17.5 kDa class I heat shock protein HSP17.8-CI	0.022
	BN17508	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)	0.045
	BN17568	similar to stress related ethylene-responsive ER6 protein	0.101
	BN18222	cold-shock DNA-binding family protein	0.357
	BN19511	universal stress protein (USP) family protein, similar to ER6 protein	0.017
	BN19515	HSF-type DNA-binding domain transcription factor	0.343
	BN19962	plant defensin-fusion protein, putative (PDF1.4)	0.056
	BN20010	similarity to drought-induced mRNA, Di19 [*Arabidopsis thaliana*]	0.367
	BN20125	dehydrin family protein	0.046
	BN20755	ABA-responsive protein (HVA22b), identical to AtHVA22b	0.191
	BN20902	identical to small heat shock protein, chloroplast precursor HSP25.3-P	0.025
	BN21105	DRE-binding protein (DREB2A) identical to DREB2A	0.041
	BN21650	DNAJ heat shock N-terminal domain-contain low similar to AHM1	0.288
	BN23037	HSF-type DNA-binding domain transcription factor	0.379
	BN23346	identical to heat shock transcription factor 6 (HSF6)	0.032
	BN24367	stress-responsive, similar to ethylene-inducible protein HEVER	0.337
	BN24377	responsive to desiccation, strong similarity to RD2 protein	0.24
	BN24919	stress-responsive protein-related to Desiccation-responsive protein 29B	0.032
	BN24925	23.6 kDa mitochondrial small heat shock protein (HSP23.6-M)	0.009
	BN24937	endomembrane-localized small heat shock protein HSP22.0-ER	0.018
	BN24959	universal stress protein (USP) family protein	0.031
	BN24975	cold-shock DNA-binding family protein / glycine-rich protein (GRP2)	0.298
	BN25006	identical to heat shock protein 101 HSP101	0.032
	BN26033	nearly identical to cold-regulated gene cor15b [*Arabidopsis thaliana*]	0.02
Group	BN11832	epoxide hydrolase, putative similar to epoxide hydrolase	0.399
2	BN27593	glycosyl hydrolase family 1 protein contains	0.068

	BN11120	expressed protein similar to auxin down-regulated protein ARG10	0.043
	BN12535	auxin-responsive protein / indoleacetic acid-induced protein 2 (IAA2)	0.259
Group	BN12888	zeaxanthin epoxidase (ZEP) (ABA1), identical to AtABA1	0.224
3	BN14718	ent-kaurene synthase / ent-kaurene synthase B (KS) (GA2)	0.31
	BN15727	identical to Ethylene responsive element binding factor 2 (AtERF2)	0.245
	BN16195	identical to ethylene-insensitive3 (EIN3)	0.341
	BN20519	identical to Gibberellin-regulated protein 3 precursor GASA 3	0.095
	BN20885	identical to Gibberellin-regulated protein 2 precursor GASA 2	0.099
	BN22832	auxin-responsive family, similar to auxin-induced protein AIR12	0.361
	BN27085	identical to ethylene receptor 1 (ETR1)	0.366
	BN10790	myb family DNA binding domain transcription factor	0.269
	BN10953	transcriptional factor B3 family protein	0.377
	BN11525	putative similar to AP2 domain transcription factor	0.312
	BN11595	cytochrome P450, putative similar to from [*Catharanthus roseus*]	0.375
	BN12119	identical to germin-like protein subfamily 3 member 3	0.399
	BN14274	similar to myb-related transcription factor (MYB15)	0.262
	BN14671	similarity to WRKY-type DNA-binding transcription factor protein	0.055
	BN16341	putative similar to AP2 domain containing protein RAP2.1	0.078
	BN17285	similarity to WRKY-type DNA-binding transcription factor protein	0.079
	BN18053	ABA-responsive element-binding protein 2 (AREB2), bZIP	0.288
	BN18567	Identical to Cytochrome P450 71B23	0.332
	BN19210	bZIP family transcription factor, similar to bZIP transcription factor	0.031
	BN19268	putative (CYP71B19), identical to cytochrome P450 71B19	0.314
Group	BN19650	strong similarity to Cuciferin CRU1 precursor	0.016
4	BN19745	WRKY family DNA -binding domain transcription factor	0.377
	BN19766	Identical to 12S seed storage protein (CRA1)	0.01
	BN21037	putative similar to cytochrome P450 89A2	0.159
	BN21176	similar to AP2 domain-containing transcription factor TINY	0.115
	BN21509	cytochrome P450 family protein	0.354
	BN21523	cytochrome P450, putative Similar to cytochrome P450 91A1	0.159
	BN21817	myb family transcription factor	0.178
	BN22223	identical to bZIP transcription factor (TGA1)	0.325
	BN22958	cytochrome P450 family protein similar to Cytochrome P450 91A1	0.35
	BN24369	putative similar to Cytochrome P450 91A1 [*Arabidopsis thaliana*]	0.05
	BN24618	identical to cytochrome P450 71B20	0.235
	BN26310	protein phosphatase 2C-related / PP2C-related protein phosphatase 2C	0.347
	BN26431	Putative similar to cytochrome P450	0.29
	BN26453	similar to putative WRKY family DNA-binding protein	0.303
	BN26536	germin-like protein, putative similar to germin -like protein GLP6	0.083
	BN26545	bZIP transcription factor family protein	0.317
	BN27443	putative (CYP71B14), identical to cytochrome P450 71B14	0.368
	BN13405	LEA3 family similar to several small proteins (~100 aa)	22.021
	BN11999	similar to DnaJ homolog subfamily B member 11 precursor	2.885
Group	BN14680	similarity to Heat shock 70 kDa protein, mitochondrial precursor	3.006
5	BN17203	putative strong similar to heat shock protein 70 [*Arabidopsis thaliana*]	2.843
	BN17226	plant defensin-fusion protein, putative (PDF2.1)	4.405
	BN17923	related to wound induced protein [Lycopersicon esculentum]	3.528
	BN21920	zinc finger (B-box type) family / salt tolerance-like protein (STH)	4.687
	BN22737	26.5 kDa class P-related heat shock protein (HSP26.5-P)	10.258
	BN13433	chitinase-like protein 1 (CTL1), similar to class I chitinase	3.544
Group	BN13522	GDSL-motif lipase/hydrolase family, similar to family II lipases EXL3	18.358
6	BN16604	GDSL-motif lipase/hydrolase family protein, similar to lipase	20.203
	BN19084	putative similar to expansin 6 (EXP6)	22.075

	BN19596	glycoside hydrolase 28/pectinase family protein	3.697
	BN19666	glycosyl hydrolase family 17, similar to elicitor inducible chitinase	2.916
	BN20239	identical to endo-1,4-beta-glucanase	5.404
Group	BN22610	glycoside hydrolase 28/pectinase family protein	2.692
6	BN22862	similar to expansin, putative EXP15	2.939
	BN23907	expansin, putative (EXP14) similar to alpha-expansin 3	5.35
	BN24511	GDSL-motif lipase/hydrolase family protein similar to Enod8.1	3.144
	BN25419	glycoside hydrolase family 28/polygalacturonase (pectinase) family	11.718
	BN25466	glycosyl hydrolase family 18 protein	2.916
	BN25913	glycosyl hydrolase family 17 protein, similar to beta-1,3-glucanase	2.908
Group 7	BN12316	1-aminocyclopropane-1-carboxylate synthase / ACC synthase	6.866
	BN14737	auxin-responsive / indoleacetic acid-induced protein 16 (IAA16)	9.527
	BN19321	putative / ACC oxidase, putative Strong similarity to ACC ox2	2.832
	BN11287	transcription factor S-II (TFIIS) domain-containing protein	3.156
	BN11437	plasma membrane intrinsic protein 2C (PIP2C) / aquaporin PIP2.3	3.372
	BN11645	similar to AP2 domain DNA-binding protein RAV2	2.85
Group	BN11710	transduction family protein / WD-40 repeat family protein	3.735
8	BN13436	luminal binding protein 1 (BiP-1) (BP1)	16.807
	BN13453	similar to luminal binding protein 2 (BiP-2) (BP2)	21.756
	BN14593	similar to protein phosphatase-2C; PP2C	2.575
	BN19599	elongation factor family protein	2.409
	BN19889	myb familyDNA-binding domain transcription factor	11.703
	BN20043	WRKY family transcription factor	3.034
	BN23109	cytochrome P450, putative similar to cytochrome P450	21.363
	BN23743	identical to bZIPDNA binding transcription factor family protein	3.37

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited by both a saline solution and ABA; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited by both a saline solution and ABA.

Table 5.8. Putative genes depressed only by a saline solution in un-germinated seeds as compared to water imbibed un-germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
	BN10330	identical to salt-tolerance zinc finger protein STZG	0.21
	BN14899	17.4 kDa class III heat shock protein (HSP17.4-CIII)	0.016
Group	BN15530	identical to Heat shock cognate 70 kDa protein 3 (Hsc70.3)	0.364
1	BN15778	dormancy/auxin associated family protein	0.064
	BN15820	identical to heat shock factor protein 7 (HSF7)	0.072
	BN17351	CCAAT-binding transcription factor (CBF-B/NF-YA) subunit B	0.31
	BN17515	identical to heat shock protein 70 [Arabidopsis thaliana]	0.372
Group 2	BN24587	glycosyl hydrolase family 17, similar to inducible chitinase Nt-SubE76	0.108
	BN25665	glycoside hydrolase family 19 protein, similar to chitinase	0.12
Group 3	BN20138	identical to auxin transport protein (BIG)	0.359
	BN15039	putative similar to 9-cis-epoxycarotenoid dioxygenase	0.013
Group	BN15391	myb family DNA-binding domain transcription factor	0.378
4	BN15587	identical to Cytochrome P450 98A3	0.181
	BN15670	cytochrome P450, similar to GB:Q05047 from [*Catharanthus roseus*]	0.377

	BN17301	AP2 domain-containing transcription factor family protein	0.392
Group	BN18656	myb family transcription factor identical to transforming protein (myb)	0.354
4	BN21157	Identical to AP2 domain-containing protein RAP2.10 (RAP2.10)	0.038
	BN24855	protein phosphatase 2C, putative / PP2C, putative	0.39
	BN25763	protein phosphatase 2C / PP2C, abscisic acid-insensitive 2 (ABI2)	0.284
	BN26892	bZIP transcription factor HBP-1b homolog	0.391
Group	BN13096	putative strong similarity to Heat shock protein 81-2 (HSP81-2)	2.91
5	BN17349	DNAJ heat shock N-terminal domain-containing protein	11.327
	BN15790	epoxide hydrolase, similar to epoxide hydrolase (*Solanum tuberosum*)	2.593
Group	BN18310	glycosyl hydrolase family 3 protein, beta-xylosidase	4.349
6	BN19352	glycosyl hydrolase family 9, similar to endo-1,4-beta glucanase	2.732
	BN23255	glycosyl hydrolase family protein 17, similar to beta-1,3-glucanase	5.481
Group 7	BN26906	gibberellin response modulator / gibberellin-responsive modulator	3.077
Group	BN10231	myb family transcription factor (KAN2)	3.364
8	BN19934	major intrinsic family protein / MIP family protein	7.567
	BN25448	myb family DNA-binding domain ,transcription factor	5.632
	BN27135	AP2 domain-containing transcription factor	3.100

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited only by a saline solution; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited only by a saline solution.

Table 5.9. Putative genes depressed only by ABA in un-germinated seeds as compared to water imbibed un-germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
	BN25166	LEA domain-containing, similarity to embryogenic abundant protein	0.068
	BN10635	universal stress protein (USP), similar to early nodulin ENOD18	0.182
	BN12218	ABA-responsive protein-related, similar to cold-induced protein kin1	0.302
	BN12619	universal stress protein (USP) family protein	0.243
	BN13104	putative strong similarity to Heat shock protein 81-2 (HSP81-2)	0.173
	BN13977	stress-inducible protein, similar to sti (stress inducible protein)	0.321
Group	BN15358	similar to Auxin-repressed, stress-related 12.5 kDa protein	0.141
1	BN15976	similarity to mitochondrial small heat shock protein	0.105
	BN16472	early-responsive to dehydration stress protein (ERD4)	0.214
	BN16483	DNAJ heat shock N-terminal domain-containing, CAJ1 protein	0.229
	BN18166	heat shock transcription factor family protein	0.038
	BN19370	identical to late-embryogenesis abundant M17 protein	0.099
	BN19713	similar to DNAJ heat shock N-terminal domain-containing CAJ1 protein	0.355
	BN26219	15.7 kDa class I-related small heat shock protein-like (HSP15.7-CI)	0.22
	BN26827	DNAJ heat shock N-terminal domain-containing protein	0.351
Group	BN18205	glycosyl hydrolase family 14 protein, similar to beta-amylase enzyme	0.362
2	BN19885	glycosyl hydrolase family 1 protein	0.204
	BN25665	glycoside hydrolase family 19 protein, similar to chitinase	0.12
Group	BN15039	putative similar to 9-cis-epoxycarotenoid dioxygenase	0.013
3	BN15777	dormancy/auxin associated family protein	0.064
	BN21637	IAA-amino acid hydrolase 3 / IAA-Ala hydrolase 3 (IAR3)	0.318

Group 4	BN10332	AP2 domain-containing transcription factor, putative	0.384
	BN14203	myb family transcription factor	0.236
	BN15980	myb family transcription factor	0.319
	BN17118	cytochrome P450, putative similar to P450 monooxygenase	0.053
	BN17160	myb family transcription factor	0.256
	BN24555	Identical to Cytochrome P450 71B5	0.082
	BN26343	protein phosphatase 2C, putative / PP2C	0.134
	BN26682	similar to Cytochrome P450 90C1 (ROTUNDIFOLIA3)	0.328
Group 5	BN10217	identical to Dehydrin ERD14 {Arabidopsis thaliana}	5.278
	BN13804	similar to early-responsive to dehydration stress ERD3 protein	2.556
	BN14084	identical to Heat shock cognate 70 kDa protein 1 (Hsc70.1)	5.83
	BN17766	universal stress protein (USP) / early nodulin ENOD18 family protein	6.721
	BN18224	cold-shock DNA-binding family protein	4.723
	BN19318	similar to early-responsive to dehydration stress ERD3 protein	8.346
	BN20254	low similarity to ERD4 protein (early-responsive to dehydration stress)	2.843
	BN21684	similar to early-responsive to dehydration stress ERD3 protein	2.512
	BN23477	related to wound-induced basic protein	2.711
	BN23491	similar to 18.0 kDa class I heat shock protein [Daucus carota]	4.969
	BN23651	similar to early-responsive to dehydration stress ERD3 protein	2.827
Group 6	BN11639	expansin, putative (EXP1) identical to expansin (At-EXP1)	40.258
	BN16591	glycosyl hydrolase family 3 protein	2.994
	BN16784	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	8.369
	BN18214	glycosyl hydrolase family 3 protein	14.508
	BN18878	expansin, putative (EXP8) similar to expansin 2	5.576
	BN18997	glycosyl hydrolase family protein 17, similar to beta-1,3-glucanase	7.456
	BN19799	alpha-xylosidase (XYL1) identical to alpha-xylosidase precursor	17.839
	BN19871	glycosyl hydrolase family 1 protein	28.134
	BN20118	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL1	7.089
	BN20878	similar to Glucan endo-1,3-beta-glucosidase precursor	6.39
	BN22296	expansin, putative (EXP10) similar to expansin At-EXP1	11.15
	BN22669	glycosyl hydrolase family 3 protein similar to beta-xylosidase	20.609
	BN23157	similar to elicitor inducible chitinase Nt-SubE76	4.551
	BN24119	beta-expansin (EXPB3), similar to soybean pollen allergen (cim1)	10.271
	BN24150	glycoside hydrolase family 28 protein / pectinase family protein	3.907
Group 7	BN11763	similar to Gibberellin-regulated protein 4 precursor GASA4;	3.092
	BN16638	nearly identical to ACC oxidase (ACC ox1) from [Brassica oleracea]	14.484
	BN16876	putative (PIN3), similar to auxin transport protein	3.787
	BN17322	auxin-responsive AUX/IAA family protein similar to IAA18	14.4
	BN21238	auxin-responsive family, similar to auxin-induced protein X10A5	17.534
	BN21365	gibberellin response modulator (RGA1)	4.317
	BN22844	similar to Gibberellin-regulated protein 1 precursor	9.614
	BN23039	identical to ethylene response sensor (ERS)	30.134
	BN23715	similar to ACC oxidase [Sorghum bicolor]	25.503
	BN26977	transcription factor MONOPTEROS (MP) / auxin-responsive IAA24	7.456
Group 8	BN10224	plasma membrane intrinsic protein 2B (PIP2B) / aquaporin PIP2.2	5.336
	BN10387	plasma membrane intrinsic protein 1B (PIP1B) / aquaporin PIP1.2	3.753
	BN12746	raffinose synthase family / seed imbibition protein, putative (din10)	39.926
	BN14121	AP2 domain-containing transcription factor RAP2.4	2.509
	BN15088	bZIP transcription factor family protein	3.098
	BN20259	plasma membrane intrinsic protein 1C (PIP1C) / aquaporin PIP1.3	6.242
	BN20556	cytochrome P450, putative	13.57
	BN23412	protein phosphatase 2C-related / PP2C-related	10.585

Group 8	BN24406	similar to myb-related transcription activator	3.908
	BN25051	putative similar to Cytochrome P450	4.137
	BN25316	fertilization-independent endosperm protein (FIE)	2.515

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited only by the ABA; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited only by the ABA.

5.3.6 A Comparison of gene expression in germinated seeds imbibed in either water, GA_{4+7}, a saline solution or ABA.

Genes with differentiation in expression greater than 2.5 fold between germinated seeds imbibed in either water, GA_{4+7}, a saline solution or ABA are shown in Figures 5.8 and 5.9. Compared to water imbibed germinated seeds, 131 genes were differentially expressed in GA treated germinated seeds, 272 for the saline solution and 641 for ABA (Figures 5.8 and 5.9.). For all the above three group genes, biggest difference occurred in SN and AN seeds, in which genes up- or down- regulated after dry seed imbibition were greatly affected compared to un-germinated seeds imbibed in water; however, after germination, this difference decreased (Figure 5.9). LEA and stress-related, hormone-related, hydrolase related and transcription factors and specific seed germination-related genes are selected and listed in Tables 5.10, 5.11, 5.12 and 5.13.

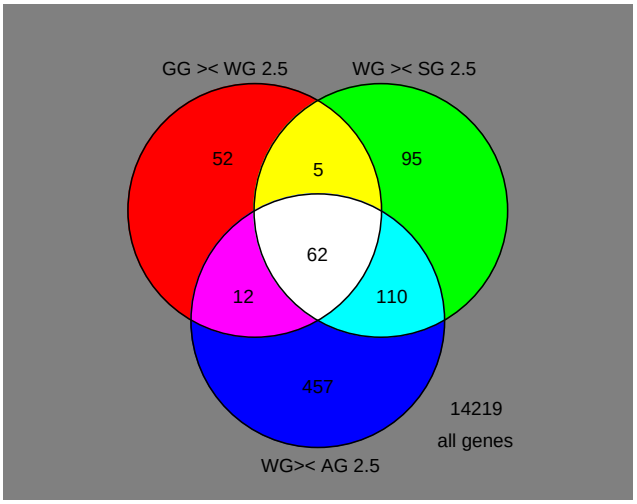


Figure 5.8. Venn diagrams for genes with differentiation in expression greater than 2.5 between germinated seeds imbibed in water and the treatments exerted. GG > < WG: GA_{4+7}; WG > < SG: a saline

solution; WG > < AG: ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

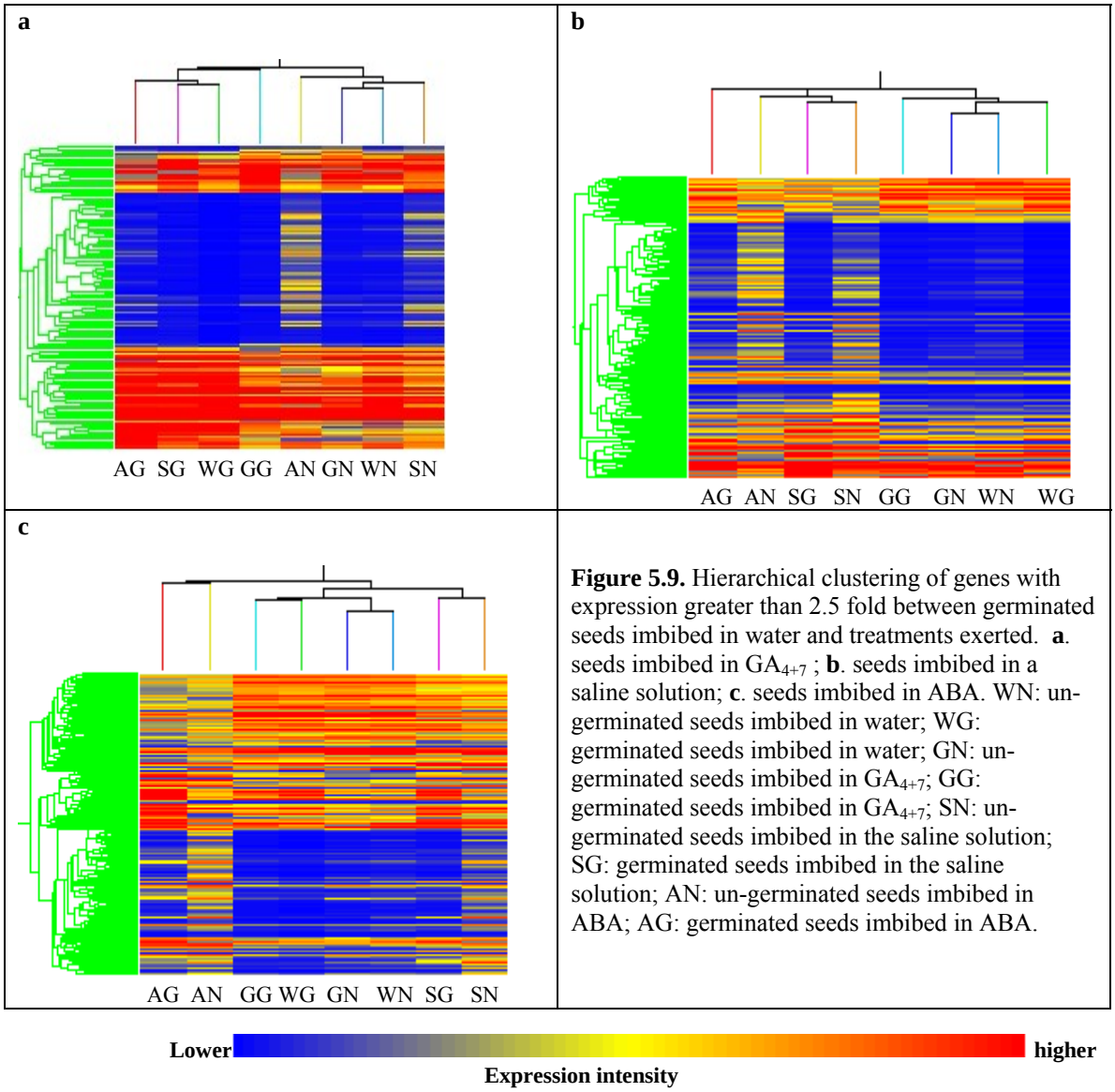


Figure 5.9. Hierarchical clustering of genes with expression greater than 2.5 fold between germinated seeds imbibed in water and treatments exerted. **a**. seeds imbibed in GA_{4+7} ; **b**. seeds imbibed in a saline solution; **c**. seeds imbibed in ABA. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

Table 5.10. Putative genes with expression differences greater than 2.5 fold between germinated seeds imbibed in water and GA_{4+7}.

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN11528	senescence/dehydration-associated protein-related (ERD7)	0.223
	BN11573	identical to dnaJ heat shock protein J11	0.286
	BN12638	putative similar to cold acclimation WCOR413-like protein	0.270
	BN12979	universal stress protein (USP) family protein similar to ER6 protein	0.352
	BN13114	1.2 kDa small heat shock family protein / hsp20 family protein	0.248
	BN18119	similar to early-responsive to dehydration stress ERD3 protein	0.354

	BN18222	cold-shock DNA-binding family protein	0.385
	BN21069	heat shock N-terminal domain-containing, low similarity to AHM1	0.371
Group 2	BN12525	glycosyl hydrolase family 1 protein	0.352
	BN16608	glycosyl hydrolase family 1 protein, .identical to beta-glucosidase	0.394
	BN24268	chitinase, putative similar to basic endochitinase CHB4 precursor	0.353
Group 3	BN24622	similar to auxin-responsive GH3 product [*Glycine max*]	0.108
Group 4	BN15915	myb family transcription factor (GLK2)	0.338
	BN17825	myb family transcription factor similar to MybSt1	0.327
	BN18317	protein phosphatase 2C, putative / PP2C	0.386
	BN18507	protein phosphatase 2C, similar to protein phosphatase type 2C	0.322
	BN18756	identical to germin-like protein subfamily 3 member 1	0.309
	BN26280	myb DNA-binding domain family transcription factor	0.374
Group 5	BN10053	similar to early-responsive to dehydration stress ERD3 protein	2.686
	BN18389	Hydrophobic RCI2B, Low temperature and salt responsive LTI6B	3.124
Group 6	BN14129	Expansin, putative (EXP5), identical to expansin At-EXP5	5.400
	BN23761	putative (EXP4) similar to alpha-expansin 6 precursor	4.031
	BN24150	glycoside hydrolase family 28/ pectinase family protein	3.906
	BN24266	chitinase, putative similar to basic endochitinase CHB4 precursor	3.816
Group 7	BN22657	auxin-responsive protein / indoleacetic acid-induced protein 4 (IAA4)	7.249
	BN25519	identical to auxin transport protein EIR1	3.486
Group 8	BN15911	myb family transcription factor (MYB30) identical to myb-like protein	25.895
	BN16235	similar to seed storage protein opaque-2(bZIP family)	2.551
	BN17150	identical to Floral homeotic protein APETALA2	4.318
	BN20382	phosphatase PP2A-3 catalytic subunit (PP2A3)	2.779
	BN20392	phosphatase 2A (PP2A) regulatory subunit B	3.676
	BN21442	myb family transcription factor	3.730

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down regulated; Group 5-8: up-regulated.

Table 5.11. Putative genes depressed by both a saline solution and ABA in germinated seeds as compared to water imbibed germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN10961	LEA group 1 domain-containing protein similarity to DS11	0.011
	BN16767	LEA protein, putative similar to Lea14-A {*Gossypium hirsutum*}	0.004
	BN20144	putative / LEA protein, similar to LEA 76 {*Brassica napus*}	0.01
	BN20349	LEA domain-containing, low similarity to Desiccation-related protein	0.002
	BN20598	LEA domain-containing protein, low similarity to D-29	0.007
	BN20688	LEA protein, nearly identical to LEA protein in group 3(ECP63)	0.019
	BN20893	LEA domain-containing, low similar to 51 kDa seed maturation protein	0.016
	BN24859	LEA group 1 domain-containing protein	0.013
	BN25011	LEA group 1 domain-containing protein	0.03
	BN25166	LEA domain-containing protein low similarity to embryogenic gene	0.004
	BN26527	putative / LEA protein, putative similar to LEA protein in group 3	0.006
	BN10431	dehydrin (RAB18), nearly identical to Dehydrin Rab18	0.004
	BN11007	related to wound-induced protein - tomato (fragment)	0.045

	BN11240	identical to dehydration-induced protein ERD15	0.205
	BN11376	putative similar to heat shock protein hsp70 from [*Pisum sativum*]	0.011
	BN11635	17.6 kDa class I small heat shock protein (HSP17.6B-CI)	0.015
	BN12218	ABA-responsive protein-related, similar to cold-induced protein kin1	0.063
	BN12279	plant defensin-fusion protein, putative (PDF1.2c)	0.015
	BN14450	identical to heat shock transcription factor 4 (HSF4)	0.356
	BN14537	putative (PDF1.2b) plant defensin protein family member	0.04
	BN14817	identical to 17.6 kDa class II heat shock protein (HSP17.6-CII)	0.007
	BN14863	identical to dormancy-associated protein, putative (DRM1)	0.201
	BN15452	identical to 17.4 kDa class I heat shock protein (HSP17.4-CI)	0.011
	BN15627	DNAJ heat shock family (Heat shock 40 kDa protein 1 homolog)	0.007
Goup	BN15648	identical to 17.6 kDa class I heat shock protein (HSP 17.6)	0.011
1	BN15778	dormancy/auxin associated family protein	0.012
	BN15854	plant defensin-fusion protein, putative (PDF2.3)	0.35
	BN17057	similar to 17.5 kDa class I heat shock protein (HSP17.8-CI)	0.015
	BN17171	low-temperature-responsive LTI78/desiccation-responsive RD29A	0.329
	BN17392	dormancy/auxin associated protein-related	0.359
	BN19511	universal stress protein (USP) family protein, similar to ER6 protein	0.017
	BN19515	HSF-type DNA-binding domain transcription factor	0.191
	BN20125	dehydrin family protein	0.007
	BN20755	ABA-responsive protein (HVA22b), identical to AtHVA22b	0.019
	BN21105	DRE-binding protein (DREB2A) identical to DREB2A	0.031
	BN24367	stress-responsive, similar to ethylene-inducible protein HEVER	0.301
	BN24377	responsive to desiccation, strong similarity to RD2 protein	0.32
	BN24919	stress-responsive protein-related to Desiccation-responsive protein 29B	0.005
	BN24925	23.6 kDa mitochondrial small heat shock protein (HSP23.6-M)	0.005
	BN24959	universal stress protein (USP) family protein	0.01
	BN25006	identical to heat shock protein 101 (HSP101)	0.011
	BN26033	nearly identical to cold-regulated gene cor15b [*Arabidopsis thaliana*]	0.01
	BN26365	DnaJ homolog subfamily B member 10	0.377
	BN27428	Identical to DRE-binding protein (DREB2A)	0.02
Group	BN15701	GDSL-motif lipase/hydrolase protein, similar to early nodulin ENOD8	0.395
2	BN25665	glycoside hydrolase family 19 protein similar to chitinase	0.282
Group	BN15907	putative similar to ethylene-responsive transcriptional coactivator	0.292
3	BN20519	identical to Gibberellin-regulated protein 3 precursor	0.023
	BN20885	identical to Gibberellin-regulated protein 2 precursor	0.011
	BN11525	putative similar to AP2 domain transcription factor	0.269
	BN11595	cytochrome P450, putative similar to from [*Catharanthus roseus*]	0.312
	BN11682	similar to cytochrome P450 from [*Catharanthus roseus*]	0.345
	BN12119	identical to germin-like protein subfamily 3 member 3	0.371
	BN15257	cytochrome P450, putative	0.046
Group	BN15698	cytochrome P450	0.286
4	BN19210	bZIP family transcription factor, similar to bZIP transcription factor	0.023
	BN19268	putative (CYP71B19), identical to cytochrome P450 71B19	0.108
	BN19650	strong similarity to Cuciferin CRU1 precursor	0.002
	BN21104	identical to basic leucine zipper transcription factor (BZIP12)	0.284
	BN21176	similar to AP2 domain-containing transcription factor TINY	0.062
	BN21509	cytochrome P450 family protein	0.383
	BN22802	cytochrome P450 family protein, similar to cytochrome P450 72A1	0.362
	BN24369	putative similar to Cytochrome P450 91A1 [*Arabidopsis thaliana*]	0.019
	BN25239	phosphatase 2C, putative / PP2C	0.308
	BN26431	Putative similar to cytochrome P450	0.318

	BN26453	similar to putative WRKY family DNA-binding protein	0.301
	BN27443	putative (CYP71B14), identical to cytochrome P450 71B14	0.298
	BN14680	strong similarity to Heat shock 70 kDa , mitochondrial precursor	2.6
Group	BN16613	nearly identical to heat shock protein hsp81.4	2.69
5	BN17203	putative strong similar to heat shock protein 70 [*Arabidopsis thaliana*]	2.709
	BN21920	zinc finger (B-box type) family / salt tolerance-like protein (STH)	4.687
	BN22737	26.5 kDa class P-related heat shock protein (HSP26.5-P)	3.237
	BN13810	glycosyl hydrolase family 1 protein	6.896
	BN19084	putative similar to expansin 6 (EXP6)	14.563
	BN19666	glycosyl hydrolase family 17, similar to elicitor inducible chitinase	3.468
	BN20239	identical to endo-1,4-beta-glucanase	4.072
	BN21244	GDSL-motif lipase/hydrolase family, similar to family II lipase EXL3	3.234
	BN21616	beta-galactosidase, putative similar to beta-galactosidase	15.223
Group	BN22579	GDSL-motif hydrolase family, low similarity to family II lipase EXL1	5.476
6	BN22610	glycoside hydrolase family 28 protein / pectinase family protein	2.574
	BN23885	glycosyl hydrolase family 35 protein similar to beta-galactosidase	5.895
	BN23907	expansin, putative (EXP14) similar to alpha-expansin 3	3.343
	BN24511	GDSL-motif lipase/hydrolase family protein similar to Enod8.1	3.57
	BN25419	glycoside hydrolase family 28/polygalacturonase (pectinase) family	12.701
	BN25913	glycosyl hydrolase family 17 protein, similar to beta-1,3-glucanase	3.085
Group	BN16411	auxin-responsive / indoleacetic acid-induced protein 17 (IAA17)	3.274
7	BN24700	auxin-responsive / indoleacetic acid-induced protein 13 (IAA13)	2.964
	BN11287	transcription factor S-II (TFIIS) domain-containing protein	3.626
	BN11710	transduction family protein / WD-40 repeat family protein	4.511
	BN17421	identical to cytochrome P450 71B28, putative (CYP71B28)	3.912
Group	BN19599	elongation factor family protein	3.735
8	BN19889	myb family transcription factor	7.376
	BN20043	WRKY family transcription factor	3.105
	BN21990	bZIP transcription factor family protein	4.967
	BN22218	myb family transcription factor	4.180
	BN22991	RNA recognition motif (RRM)-containing protein	7.962
	BN25316	fertilization-independent endosperm protein (FIE)	2.710

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited by both a saline solution and ABA; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited by both a saline solution and ABA.

Table 5.12. Putative genes depressed only by a saline solution in germinated seeds as compared to water imbibed germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
	BN19370	identical to late-embryogenesis abundant M17 protein	0.017
	BN19387	late embryogenesis abundant protein (M10) / LEA protein M10	0.004
Group	BN11573	identical to dnaJ heat shock protein J11	0.394
1	BN13968	putative (PDF1.2a) plant defensin protein family member	0.044
	BN14899	17.4 kDa class III heat shock protein (HSP17.4-CIII)	0.019
	BN15189	low similarity to 40 kDa heat shock chaperone protein (HSP40)	0.209
	BN17568	similar to ethylene-responsive ER6 protein	0.058
	BN18222	cold-shock DNA-binding family protein	0.247
	BN19713	DNAJ heat shock N-terminal domain-containing protein	0.285

	BN19962	putative (PDF1.4) plant defensin protein family member	0.01
	BN23037	HSF-type DNA-binding domain transcription factor	0.355
	BN23186	similar to drought-induced mRNA, Di19	0.364
	BN23509	universal stress protein (USP), similar to early nodulin ENOD18	0.288
	BN24993	expressed protein very low similarity to LEA protein	0.042
	BN26469	defense protein-related weak similarity to RPM1-interacting protein 4	0.201
Group 2	BN18357	hydrolase, alpha/beta fold family protein	0.03
	BN19885	glycosyl hydrolase family 1 protein contains	0.064
	BN26772	glycosyl hydrolase family 1 protein contains	0.165
	BN27593	glycosyl hydrolase family 1 protein	0.272
Group 3	BN15727	identical to Ethylene responsive element binding factor 2 (AtERF2)	0.225
	BN21637	IAA-amino acid hydrolase 3 / IAA-Ala hydrolase 3 (IAR3)	0.255
	BN22832	auxin-responsive family, similar to auxin-induced protein AIR12	0.32
Group 4	BN15391	myb family DNA-binding domain transcription factor	0.373
	BN15670	cytochrome P450	0.363
	BN18118	myb family transcription factor	0.357
	BN18567	Identical to Cytochrome P450 71B23	0.365
	BN18656	myb family transcription factor identical to transforming protein (myb)	0.257
	BN19766	identical to 12S seed storage protein (CRA1)	0.002
	BN22958	cytochrome P450 family protein, similar to Cytochrome P450 91A1	0.368
	BN26536	germin-like protein, putative similar to germin -like protein GLP6	0.05
	BN26545	bZIP transcription factor family protein	0.189
Group 5	N/A	N/A	N/A
Group 6	BN15161	glycosyl hydrolase family 3 protein	23.897
Group 7	N/A	N/A	N/A
Group 8	BN14593	similar to protein phosphatase-2C; PP2C	3.012
	BN16022	myb family transcription factor	2.783
	BN19934	major intrinsic family protein / MIP family protein	7.072
	BN24459	WRKY family transcription factor	3.361
	BN24616	cytochrome P450, putative	2.796

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited by a saline solution and ABA; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited only by a saline solution.

Table 5.13. Putative genes depressed only by ABA in germinated seeds as compared to water imbibed germinated seeds (with expression differences greater than 2.5 fold).

Groups	Clone ID	Gene Names	Relative Ratio
Group 1	BN19387	late embryogenesis abundant protein (M10) / LEA protein M10	0.004
	BN10459	similar to wound inducive gene	0.21
	BN10635	universal stress protein (USP), similar to early nodulin ENOD18	0.093
	BN10668	similar to Chaperone protein dnaJ Erysipelothrix rhusiopathiae	0.034

	BN12619	universal stress protein (USP) family protein	0.088
	BN12981	universal stress protein (USP) family protein similar to ER6 protein	0.035
	BN13289	nearly identical to stress enhanced protein 2 (SEP2)	0.121
	BN14532	DNAJ heat shock N-terminal domain-containing DnaJ protein Tid-1	0.362
	BN16309	weak similarity to HSF 1 (Heat shock transcription factor 1) (HSTF 1)	0.235
	BN16590	similar to early-responsive to dehydration stress ERD3 protein	0.384
	BN19962	putative (PDF1.4) plant defensin protein family member	0.01
	BN21650	DNAJ heat shock N-terminal domain-containing AHM1 protein	0.26
	BN26219	15.7 kDa class I-related small heat shock protein-like (HSP15.7-CI)	0.341
Group 2	BN18758	hydrolase, low similarity to monoglyceride lipase from [*Homo sapiens*]	0.133
	BN21637	identical to IAA-Ala hydrolase (IAR3) [Arabidopsis thaliana]	0.255
	BN26438	GDSL-motif lipase/hydrolase family protein similar to lipase	0.269
Group 3	BN12888	zeaxanthin epoxidase (ZEP) (ABA1), identical to AtABA1	0.125
	BN15358	similar to Auxin-repressed 12.5 kDa protein	0.131
	BN24622	similar to auxin-responsive GH3 product	0.344
Group 4	BN10790	myb family DNA binding domain transcription factor	0.307
	BN10953	transcriptional factor B3 family protein	0.343
	BN13703	protein phosphatase 2C, putative / PP2C	0.376
	BN16242	protein phosphatase 2C, putative / PP2C from [Arabidopsis thaliana]	0.204
	BN17160	myb family transcription factor	0.255
	BN22940	WRKY family transcription factor	0.381
	BN22954	protein phosphatase 2C P2C-HA / PP2C P2C-HA	0.193
	BN25156	similar to ABA-responsive element binding protein 1 (AREB1) bZIP	0.121
	BN25763	protein phosphatase 2C / PP2C, abscisic acid-insensitive 2 (ABI2)	0.22
	BN26682	similar to Cytochrome P450 90C1 (ROTUNDIFOLIA3)	0.215
Group 5	BN13405	LEA3 family protein similar to several small proteins (~100 aa)	17.471
	BN11999	similar to DnaJ homolog subfamily B member 11 precursor	3.892
	BN13096	putative strong similarity to Heat shock protein 81-2 (HSP81-2)	3.169
	BN14084	identical to Heat shock cognate 70 kDa protein 1 (Hsc70.1)	10.212
	BN18224	cold-shock DNA-binding family protein	2.578
	BN23491	similar to 18.0 kDa class I heat shock protein [Daucus carota]	3.63
	BN23651	similar to early-responsive to dehydration stress ERD3 protein	2.52
Group 6	BN14925	beta-1,3-glucanase (BG3) almost identical to beta-1,3-glucanase	4.048
	BN15630	alpha-glucosidase, putative similar to alpha-glucosidase	2.963
	BN16591	glycosyl hydrolase family 3 protein	2.836
	BN18878	expansin, putative (EXP8) similar to expansin 2	4.696
	BN18997	glycosyl hydrolase family protein 17, similar to beta-1,3-glucanase	2.926
	BN20681	expansin family protein (EXPL2)	4.496
	BN20878	similar to Glucan endo-1,3-beta-glucosidase precursor	4.124
	BN21978	GDSL-motif lipase/hydrolase family protein	3.262
	BN22148	similar to glucan endo-1,3-beta-glucosidase	7.024
	BN23157	similar to elicitor inducible chitinase Nt-SubE76	3.302
	BN26929	GDSL-motif lipase/hydrolase family, similar to family II lipases EXL3	42.281
Group 7	BN21238	auxin-responsive family, similar to auxin-induced protein X10A5	3.367
	BN21365	gibberellin response modulator (RGA1)	2.659
	BN26977	transcription factor MONOPTEROS (MP) / auxin-responsive IAA24	2.57
Group 8	BN14121	AP2 domain-containing transcription factor RAP2.4	2.57
	BN14194	myb family transcription factor	3.232

Group 8	BN15837	myb family transcription factor	2.929
	BN23109	cytochrome P450, putative similar to cytochrome P450	17.74

Four specific group genes as described in table 5.2 were selected. Group 1-4: Down-regulated genes for seeds imbibed in water were inhibited only by ABA; Group 5-8: Up-regulated genes for seeds imbibed in water were inhibited only by ABA.

5.3.7 Genes, as described in Table 5.2, specifically up-regulated by either a saline solution or ABA

Compared to the water imbibed seeds as described in Table 5.2, we also found some genes up-regulated more in seeds imbibed in either the saline solution or ABA. Genes and expression profiles are displayed in Tables 5.14, 5.15 and Figures 5.10 and 5.11. We assumed that these genes were involved in overcoming the inhibitory effects generated by either the saline solution or ABA.

Table 5.14. Genes specifically up-regulated in seeds imbibed in a saline solution.

Colne ID	Gene name
BN12534	auxin-responsive protein / indoleacetic acid-induced protein 2 (IAA2)
BN13944	early-responsive to dehydration stress protein (ERD3) identical to ERD3 protein
BN18296	early-responsive to dehydration stress (ERD6) / sugar transporter family protein
BN20099	expansin family protein (EXPR3) identical to Expansin-related protein 3 precursor
BN20385	AP2 domain-containing transcription factor RAP2.7 (RAP2.7)
BN22148	glycosyl hydrolase family 17 protein similar to glucan endo-1,3-beta-glucosidase
BN22657	auxin-responsive/ indoleacetic acid-induced protein 4 (IAA4) / auxin-induced protein
BN25050	cytochrome P450, putative similar to Cytochrome
BN25697	transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related
BN26349	putative (CYP71B22) Identical to cytochrome P450 71B22
BN27674	protein phosphatase 2C ABI1 / PP2C ABI1 / abscisic acid-insensitive 1 (ABI1)

a

b

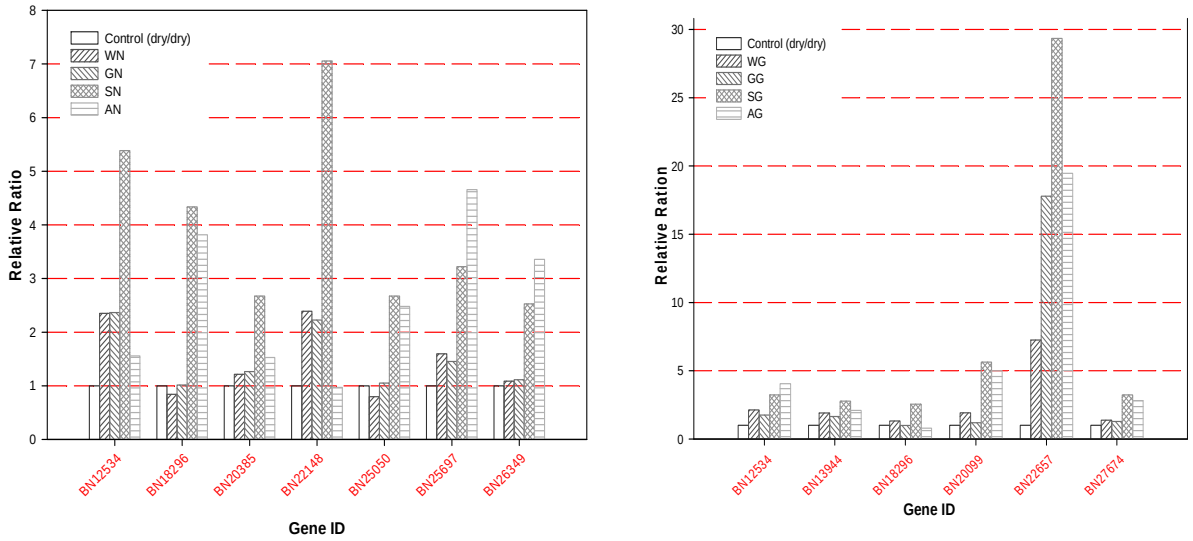
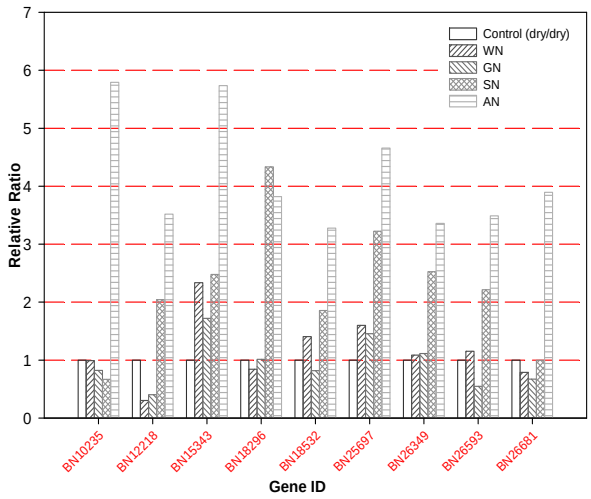


Figure 5.10. Compared to seeds imbibed in water, genes specifically up-regulated in seeds imbibed in the saline solution. a. un-germinated seeds; b. germinated seeds. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

Table 5.15. Genes specifically up-regulated in seeds imbibed in ABA.

Colne ID	Gene name
BN10195	dehydrin (COR47) identical to dehydrin COR47 (Cold-induced COR47 protein)
BN10235	superoxide dismutase [Cu-Zn], chloroplast (SODCP) /
BN10799	calcium-binding RD20 protein (RD20) induced by abscisic acid during dehydration
BN10936	identical to dehydrin ERD10 (Low-temperature-induced protein LTI45)
BN11835	stress-responsive protein (KIN2) / cold-responsive protein (COR6.6)
BN12218	ABA-responsive protein-related similar to ABA-inducible protein
BN12534	auxin-responsive protein / indoleacetic acid-induced protein 2 (IAA2)
BN15343	putative (FL3-5A3) similar to cold acclimation WCOR413-like protein
BN17214	low-temperature-responsive 65 kD (LTI65) / desiccation-responsive 29B (RD29B)
BN17472	aquaporin, putative similar to plasma membrane aquaporin 2b
BN18296	early-responsive to dehydration stress (ERD6) / sugar transporter family protein
BN18389	hydrophobic protein (RCI2B) / low temperature and salt responsive protein (LTI6B)
BN18532	cytochrome P450 84A1 (CYP84A1) / ferulate-5-hydroxylase (FAH1)
BN23484	identical to WRKY transcription factor 31 (WRKY31)
BN25697	transcriptional factor B3 family protein / auxin-responsive factor AUX/IAA-related
BN26221	myb family transcription factor (MYB47)
BN26349	putative (CYP71B22) Identical to cytochrome P450 71B22
BN26593	putative (CYP71A16) Identical to Cytochrome P450 71A16
BN26681	cytochrome P450 family, similar to Cytochrome P450 90C1 (ROTUNDIFOLIA3)

a



b

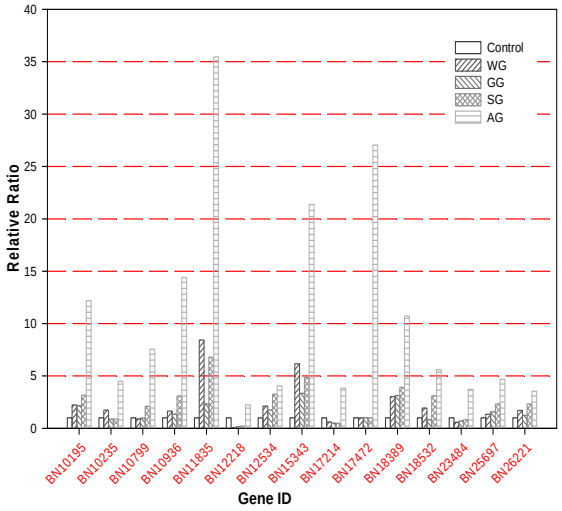


Figure 5.11. Compared to seeds imbibed in water, genes specifically up-regulated in seeds imbibed in ABA. a. un-germinated seeds; b. germinated seeds. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA.

5.3.8 Northern blot analysis:

Fragments of two new LEA genes ($D_1$400 and $D_2$600), isocitrate lyase (IS) and malate synthase (MS) genes from canola were cloned and their expression were analyzed by northern blot analysis.

RT-PCR results of $D_1$400 and $D_2$600

Based on the conserve motif of LEA genes, we designed two pairs of degenerate primers and isolated two new LEA gene fragments from canola seed.

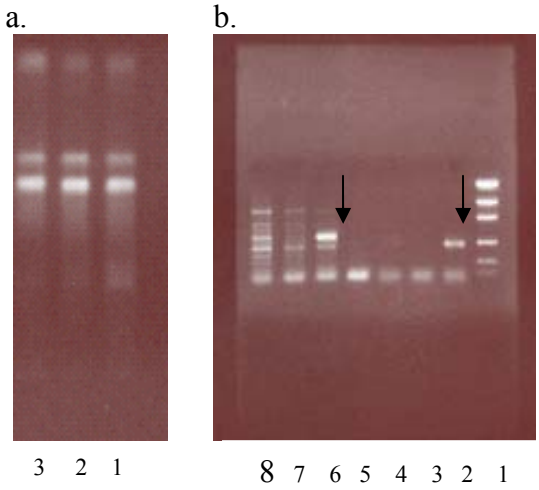


Figure 5.12. RT-PCR results for two new LEA genes. a. Quantitative RNA samples (1µg). 1-3 are the RNA from dry seeds and seeds imbibed in water and ABA, respectively. b. RT-PCR results using these two degenerate primers. 1: marker; 2-4 (first set primer): dry seeds, seeds imbibed in water and ABA, respectively; 5: Control; 6-7 (second set primer): dry seeds, seeds imbibe in water and ABA respectively.

Gene fragments (as arrows indicate in Figure 5.12 b) revealed by these two primers which had different expression in dry seeds and seeds imbibed in water and ABA respectively, were cloned. First band, $D_1$400 has 400 bp and is homologous the Arabidopsis *RAB18* gene; the second band, D_2 600 which has 478 bp is closely to the *Brassica napus* dehydrin gene.

Northern blot analysis of IS, MS, $D_1$400 and $D_2$600 gene expression

Expression of these four genes in both germinated (WG, GG, SG and AG) and un-germinated (WN, GN, SN, AN) seeds at 50% germination imbibed in water (WN and WG), GA_{4+7} (GN, GG), the saline solution (SN, SG) and ABA (AN, AG) at 8°C is shown in Figure 5.13.

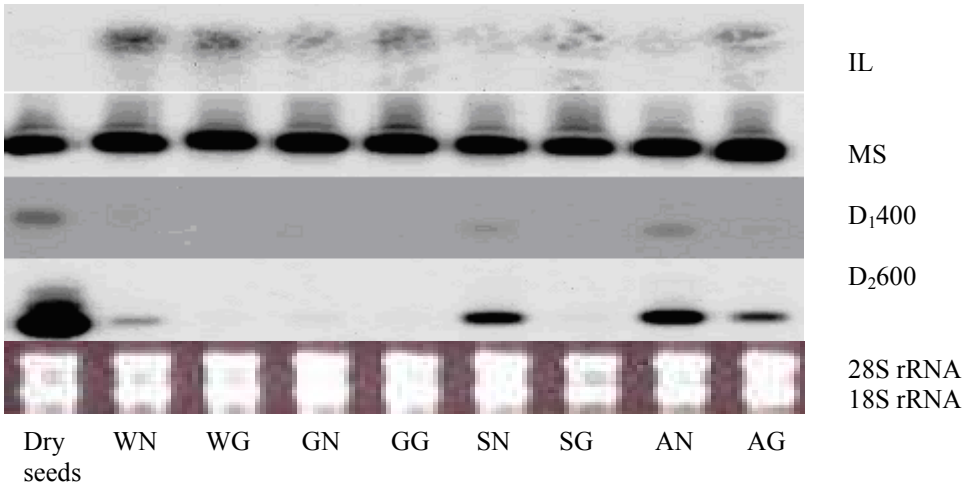


Figure 5.13. Expression analysis of genes IS, MS, D_1400 and D_2600. WN: un-germinated seeds imbibed in water; WG: germinated seeds imbibed in water; GN: un-germinated seeds imbibed in GA_{4+7}; GG: germinated seeds imbibed in GA_{4+7}; SN: un-germinated seeds imbibed in the saline solution; SG: germinated seeds imbibed in the saline solution; AN: un-germinated seeds imbibed in ABA; AG: germinated seeds imbibed in ABA

These results suggest that IL and the two LEA genes (D_1400, D_2600) are associated with seed germination, specially, D_2 600. The expression of MS was not affected by any of the treatments.

Gene expression profiles of D_1400 and D_2600 during seed imbibition

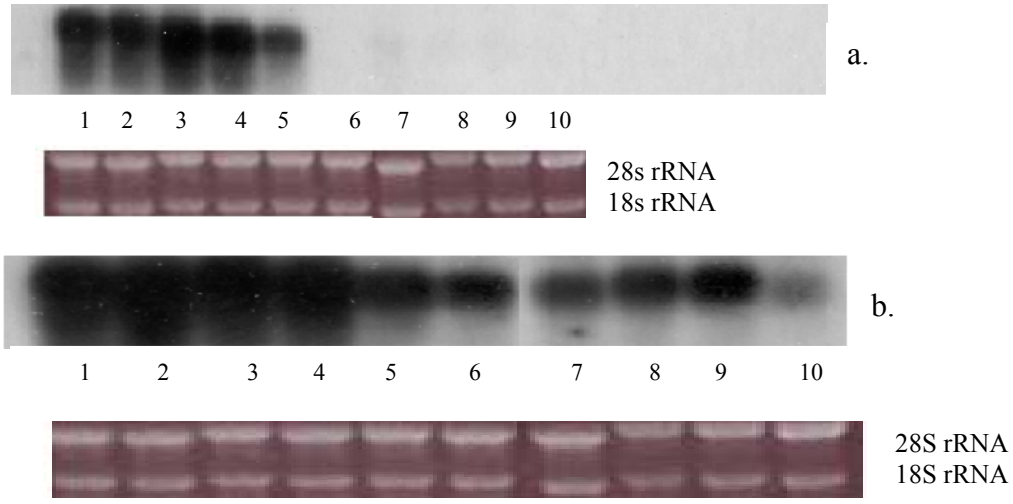


Figure 5.14. Gene expression profiles of D_1400 and D_2 600 for seeds imbibed in water at 8 ºC. Lane 1: dry seeds; Lane 2-9: un-germinated sees imbibed in water for 4, 8, 12, 24, 36, 48, 60 and 72 hours, respectively; Lane 10: germinated seeds imbibed in water for 72 hours.
a. D_1400; b. D_2600.

D_1400 declined but was still present at 24h of imbibition, whereas, D_2600 degraded much slower over the 72h period for seeds imbibed at 8 ºC in water. Higher level of D_2600 was still present after 72 h in un-germinated seeds. These results indicate that D_2600 is closely associated with seed germination.

Gene expression of D_2600 in seeds from four canola lines that differ in germination at 8ºC

Table 5.16: Germination responses of four seed lines that differ in germination Potential.

Lines	Germination%, 5d, 8°C, water	Germination (%), 5d, 8°C, in saline solution
A	96.0	51.5
B	99.0	94.8
E	85.3	12.8
K	94.3	14.3

Northern blot analysis

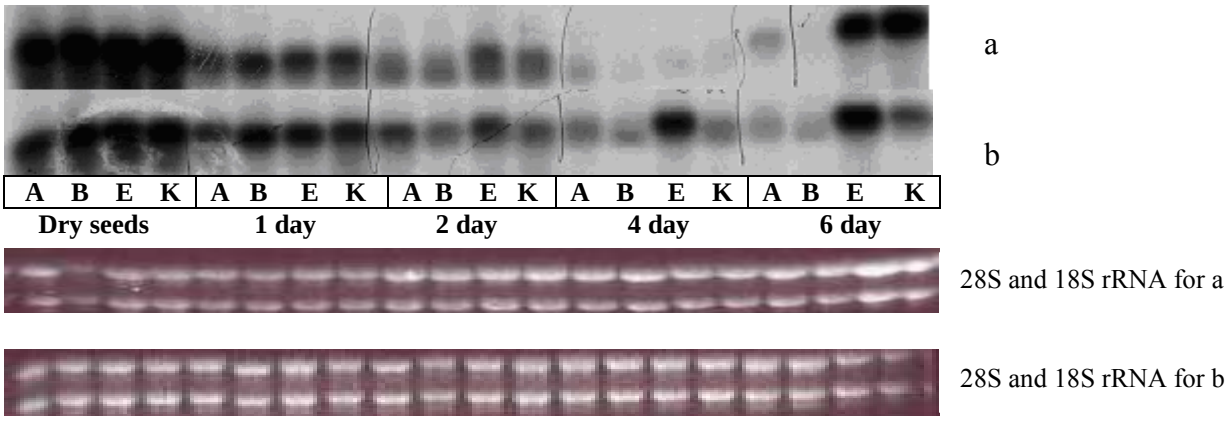


Figure 5.15. D_2600 expression in four seed lines imbibed at 8°C in either water or 80 mM saline solution for 1, 2 4 and 6 days. a. Water; b. 80 mM saline solution.

From the above results, we found that D_2600 expression was associated with seed germination at 8 ºC. Good germination performance lines had lower expression of this gene and poor performance lines had higher expression.

5.4 Discussion

5.4.1 Gene expression profiles at different physiological states of germination

Previous studies have established that seed germination potential is controlled by stored mRNA in dry seeds, while the rate of germination is controlled by changes of gene expression initiated upon imbibition (Holdsworth et al., 2008; Bentsink and Koornneef, 2002; Rajjou et al., 2004). However, it is not clear how the environment controls or regulates seed germination in these studies. In addition, our understanding of this process primarily comes from studies on Arabidopsis. In this study, we investigated the effect of GA_{4+7}, saline solution and ABA on gene expression in both germinated and un-germinated canola seeds, which is an important crop in western Canada and is also closely related to Arabidopsis.

When seeds reached 50% germination, gene expression profiles of germinated and un-germinated seeds for all treatments were analyzed by principle component analysis (PCA). The PCA analysis separated the eight seed samples into different groups based on the similarity of their gene expression pattern. This gene expression pattern classification was consistence with the seed germination performance displayed in Figure 5.1. The gene expression profiles for seeds imbibed in water and GA_{4+7} were similar, whereas, this similarity for seeds imbibed in either the saline solution or ABA which delayed the rate of germination decreased. There were less differences between germinated and un-germinated seeds imbibed in either water or GA_{4+7} as compared to seeds imbibed in either the saline solution or ABA by PCA analysis. Results from the gene hierarchical clustering also verified these differences. All the results suggested that gene expression profiles in seeds are involved in seed germination.

Gene expression patterns between germinated and un-germinated seeds for each treatment were selected and clustered for all treatments. Based on the similarity of the gene expression, un-germinated seeds could be separated into two groups: WN, GN and AN, SN. Gene expression pattern was similar within groups; however, obvious differences occurred between groups. In germinated seeds, the differences between groups were greatly reduced in the four treatments. Although there were clear differences in gene expression between un-germinated and germinated seeds, the least differences were observed in seeds imbibed in water and GA and highest for seeds imbibed in either the saline solution or ABA. These results suggest certain genes are up-regulated and down-regulated earlier in water or GA imbibed seeds compared to the saline solution or ABA treated seeds. The gene expression pattern between un-germinated and germinated seeds indicates that specific genes are strongly associated with seed germination performance.

The gene expression profiles of seeds imbibed in either ABA or the saline solution was very similar indicating some common regulation. A comparison of gene expression for seeds imbibed in the saline solution and ABA revealed several differences, suggesting different mechanism of germination inhibition.

To investigate this specific gene expression program, we picked four major group genes: *LEA* and stress related, hormone related; hydrolase related; and specific seed germination related genes.

5.4.2 *LEA* and stressed related genes

Late embryogenesis abundant (LEA) proteins characterized by a strong degree of hydrophilicity and boiling stable are synthesized in late stages of seed maturation (dry

down stage) and disappear during germination (Ingram and Bartels, 1996; Cuming, 1999). These proteins were proposed to play protective roles in seed desiccation process (Ingram and Bartels, 1996; Cuming, 1999). Although LEA proteins are important to protect dry seeds, their function in seed germination is unclear. In our studies, we found LEA genes displayed different expression pattern depending upon the treatment. One group of LEA transcripts decreased in all treatments whether the seeds germinated or not. This group of LEA genes may only function in the seed maturation process and have no function in seed germination. In comparing imbibed un-germinated seeds to germinated seeds, one group of LEA transcripts were down-regulated more in germinated seeds. This suggests that down-regulation of these LEA genes is required for the seed to germinate. Another group of LEA genes, although down-regulated compared to dry seeds, had higher expression levels in SN and AN seeds than WN seeds. Seeds imbibed in water germinate faster than seeds imbibed in either the saline solution or ABA therefore, expression of these LEA genes appears to be negatively associated with germination. We also demonstrated that fast germination canola lines have lower level of a specific LEA transcript than the slow germination lines which suggests these transcripts are degraded faster in good germination lines or they are somehow involved in germination. However, several studies revealed LEA genes were positively associated with germination of seeds subjected to stressful conditions, which is somewhat contradictory to our conclusions (Soeda et al., 2005). We can hypothesize that down-regulation of specific LEA genes is required for the initiation of seed germination and the rate of this degradation dictates the seed germination rate; however, down-regulation of LEA genes reduce seed vigour to adverse conditions. The poor germination rate of

primed seeds versus un-primed seeds subjected to controlled deterioration or accelerated aging may be in part due to the down regulation of LEA genes (Soeda et al., 2005). Rajjou et al. (2006) found that salicylic acid stimulated Arabidopsis seed germination under salt conditions, whereas, under optimal conditions, seed germination was inhibited. Proteomic analysis also revealed there were higher levels of LEA proteins in salicylic acid imbibed seeds compared to water imbibed seeds. Higher levels of LEA proteins were also observed in seeds treated with α-amanitin, an inhibitor targeting RNA polymerase Ⅱ (Rajjou et al., 2004). After imbibition, higher levels of LEA proteins were observed in dormant Arabidopsis seeds compared to non-dormant seeds as well as in ABA treated non-dormant seeds compared to control seeds (Chibani et al., 2006). From the above results, we propose that during seed germination, certain LEA genes may be involved in controlling germination rate. In the early stage of Arabidopsis germination, seed can recapitulate its maturation program if water becomes limiting to protect seeds from this stress (Lopez-Molina et al., 2001, 2002). Therefore, during seed germination, re-induction of some LEA genes which express at seed maturation stage would be a protective mechanism for seeds to deal with adverse conditions. From above results, it appears that certain LEA genes play a role in seed maturation program; however, certain LEA transcripts may also affect seed germination.

In addition to the LEA genes, some stress-related genes were up-regulated during imbibition and had higher expression in germinated seeds than un-germinated seeds. For example, Bn10217, a gene homologous to *ERD14* (early response to dehydration) (Alsheikh et al., 2003) was highly expressed in WG, GG, SG and AG seed compared to their un-germinated seeds (WN, GN, SN and AN) respectively. This group of genes may

be required for the seed to germinate at low temperature. BN 10195, homologous to dehydrin *COR47* (Yamaguchi-Shinozaki and Shinozaki, 2006), was only up-regulated in ABA or the saline solution imbibed seeds, suggesting a role for this group of genes may be involved in stress.

ERD6 (early response to dehydration) is induced in by cold and dehydration stress in Arabidopsis (Kiyosue et al., 1998); however, this gene was only up-regulated in SN and AN seeds. Possibly, *ERD6* inhibits germination of seeds exposed to stress. Higher levels of ABA generally indicate plants are exposed to a stress (Leung and Giraudat, 1998). Therefore, *ERD6* may prevent germination when environmental conditions are adverse. Bn15343, homologous to the cold acclimation gene *WCOR413* which is induced by low temperatures (Breton et al., 2003), was up-regulated in all the imbibed seeds. The highest level of expression was observed in AN and AG seeds. These results suggest that BN15343 is also induced by low temperatures in seeds and ABA had an additive effect on its expression. *WCOR413* has been proposed to enhance freezing tolerance in Arabidopsis and cereal plants by stablizing the bilayer plasma membrane or acting in the stress signal transduction pathway (Breton et al., 2003). This gene may also have a similar role for seed germination at low temperatures. BN12218, homologous to ABA inducible genes, was only induced in seeds imbibed in ABA and down-regulated in other seeds, indicating this gene may be involved in ABA signal transduction. Compared to water imbibed seeds, other genes involved in ABA signal transduction such as *RD20*, *ERD10*, *COR* and *RD29B* (Yamaguchi-Shinozaki and Shinozaki, 2006), were also up-regulated in seeds imbibed in ABA. ABA, a stress hormone, has been proposed to activate the plant resistant machine to enhance the abiotic stress tolerance (Nambara and

Marion-Poll, 2005). Zheng et al. (1994) reported that canola seed primed with ABA had a more rapid and uniform germination rate compared to seeds primed with water. In our results, these stressed related genes up-regulated by ABA may be involved in enhancing seed germination by ABA priming.

5.4.3 Hormone related genes

GA and ABA are proposed to be the two major hormones in controlling seed germination: GA stimulates seed germination and ABA inhibits seed germination (Kucera et al., 2005). Studies on the metabolism of GA and ABA revealed that the anabolism and catabolism of GA and ABA, which are controlled by distinct pathways affect seed germination (Hedden and Kamiya, 1997; Nambara and Marion-Poll, 2005). The ability of exogenous GA to completely restore germination of GA deficient mutants or a GA inhibitor, paclobutrazol revealed GA synthesis is required for germination (Koornneef and van der Veen, 1980; Groot and Karssen, 1987; Karssen et al., 1989; Nambara et al., 1991). In Arabidopsis, the GA biosynthesis pathway is divided into three stages in which GA 20-oxidase is involved into the last stage (Lange, 1998; Pérez-Flores et al, 2003). A gene BN24370, homologous to gibberellin 20-oxidase gene, was identified to be up-regulated more in germinated seeds as compared to un-germinated seeds for all treatments. The expression pattern of GA 20-oxidase gene is consistent with the results found in Arabidopsis where GA was mainly involved in the later stage of seed germination (Ogawa et al., 2003; Yamauchi et al., 2004).

Zeaxanthin epoxidase (ZEP/ABA1) gene which is involved in ABA synthesis (Koornneef et al., 1982; Nambara and Marison-Poll, 2005) was down-regulated more in SG and AG seeds compared to their un-germinated counterparts, while no difference was

detected in WG and GG seeds compared to WN and GN seeds. *ZEP/ABA1* expression was higher in SN and AN seeds compare to WN seeds, while in WG and SG seeds, it was equivalent in expression. For AG seeds, its expression was still higher than in WG seeds. Mutations of *ZEP* have been shown to reduce seed dormancy (Koornneef et al., 1982; Nambara and Marison-Poll, 2005). Over-expression of *NpZEP* in *Nicotiana plumbaginifolia* delayed seed germination which in part is attributed to a higher level of ABA (Frey et al., 1999). Toh et al. (2008) reported that the inhibitory effect of high temperatures on Arabidopsis seed germination is associated with reduced down-regulation of *ZEP/ABA1*. The expression pattern of *ZEP/ABA1* in our studies clearly indicate that down-regulation of this gene is associated with seed germination.

In our studies, ethylene related genes are also involved in the seed germination. Ethylene has been proposed to promote seed germination in some species (Kepczynski and Kepczynska, 1997; Matilla, 2000; Kucera et al., 2005). ACC, the precursor of ethylene is produced by ACC synthases and then ACC is oxidized to ethylene by ACC oxidases (Barry et al., 1996; Bouquin et al., 1997; Oetiker et al., 1997). In studies on chick pea seed germination, ACC oxidase was up-regulated just before the emergence of the radicle (Petruzzelli et al., 2000, 2003). From our results, a putative ACC oxidase gene, *ACCox2* and an ACC synthase gene were up-regulated more in SG and AG seeds, whereas in SN and AN seeds, the transcripts of these two genes were at same levels as dry seeds. These two genes were up-regulated similarly in both germinated and un-germinated seeds imbibed in either water or GA_{4+7}. The levels of expression of *ACCox2* and ACC synthase gene in SN and AN seeds were lower compared to WN seeds; however, the levels of expression were similar in WG, GG, SG and AG seeds. This

suggests that up-regulation of *ACCox2* and ACC synthase gene plays a role in the germination of canola seeds at 8ºC. We also found another ACC oxdiase gene, *ACCox1*, which was only inhibited in AN seeds, whereas it was up-regulated similarly in WN, WG, GN, GG, SN, SG and AG seeds compared to dry seeds. Possible, *ACCox1* is involved in ABA inhibited seed germination. In addition, results from the above studies provide evidence that ethylene and ABA play an antagonistic role in the seed germination as has been suggested previously (Kucera et al., 2005).

5.4.4 Hydrolase related genes

Hydrolases and other cell wall proteins including endo-β-1, 3-glucanase, chitinase, endo-β-mannanase and expansin, are involved in weakening the cell walls or seed coat (testa) to facilitate radicle emergence (Ikuma and Thiman, 1963; Wu et al., 2001; Nonogaki et al., 2000; Chen and Bradford, 2000; Dubreucq et al., 2000). We observed during imbibition, hydrolase genes were up-regulated more in geminated seeds than in un-germinated seeds, especially for ABA treated seeds which had the largest number of the hyrolase genes differently expressed in AG versus AN seeds. Gibberellin has been proposed to stimulate seed germination by inducing hydrolase related genes to lower the mechanical resistance of the seed coat (Debeaujon and Koornneef, 2000; Leubner-Metzger, 2003). An expansin gene, *At-EXP5* was specifically induced in GA imbibed seeds compared to water imbibed seeds. Also, an expansin gene, *LeEXP4* which is GA inducible, was found to be involved in seed germination process by weakening the endosperm in tomato (Chen and Bradford, 2000). Compared to WN seeds, BN13433, a chitinase-like protein 1 (CTL1) gene, was not up-regulated in SN and AN seeds, whereas, similar levels of expression occurred in WG, GG, SG and AG seeds. In tomato, chitinase

accumulates just before radicle emergence and is not affected by exogenous ABA (Wu et al., 2001). In Arabidopsis, a CTL1 gene is involved in the heat, salt and drought tolerance of seedling (Kwon et al., 2007). In our study, up-regulation of CTL1 gene did not occur in SN and AN seeds, but in WN and GN seeds, which suggests it may be involved in enhancing seed germination rate and is required for the late phase of seed germination. A hydrolase gene, endo-β-1,4 glucanase gene was up-regulated in both un-germinated and germinated seeds imbibed in either water or GA; however, the level of transcripts was depressed in seeds imbibed in either the saline solution or ABA. This group of genes may be not essential, but can enhance the seed germination rate.

5.4.5 Specific genes related to seed germination

Included in this group, are specific transcription factors, phosphatases, aquaporins which demonstrated differential expression patterns depending on the seed treatments.

Studies on ABA insensitive mutants *abi1-1* and *abi1-2* which were able to germinate in the presence of ABA which was inhibitory to wild type seeds (Koornneef et al., 1984) revealed that *ABI1* and *ABI2* encoded two type of 2C phosphatases (PP2Cs) (Leung et al. 1994, 1997; Rodriguez et al. 1998). The *abi1-2* mutation is dominant and ABI1 type PP2Cs play negative roles in ABA signal responses (Leung and Giraudat et al., 1998; Sheen, 1998; Beaudoin et al., 2000; Schweighofer et al., 2004). However, over-expression and microinjection of *ABI1* did not block ABA signal transduction (Wu et al., 2003). We observed that *ABI1* was up-regulated only in SG and AG seeds. *ABI1* may be involved in overcoming the inhibitory effect of the saline solution and ABA. Similar results were obtained by Leung et al. (1997) studying the effect of ABA and sorbitol on

the germination of Arabidopsis. Unfortunately the role of ABI1 in ABA signaling, is still unclear, which made it difficult to interpret its role in seed germination.

As stated earlier, germination is initiated during imbibition and is considered complete when the radicle penetrates the seed coat or testa (Bewley and Black 1994, Bewley 1997a). To enable radicle protrusion, water uptake and cell wall weakening play important roles in this process (Bewley, 1997b; Koornneef et al., 2002; Manz et al., 2005). Water uptake is characterized as a triphasic process with a rapid initial water uptake (driven by physical forces) followed a plateau phase (associated with the resumption of metabolism) and then, with the occurrence of another increase in water uptake (mainly associated with the radicle protrusion) (Bewley, 1997a). Studies on water uptake by nuclear magnetic resonance (NMR) spectroscopy revealed that water distribution is under precise control which is involved in seed germination (Krishnan et al., 2004; Manz et al, 2005). Molecular studies have been shown that aquaporins play important roles in the water uptake and transport in plants (Maurel et al., 2001). Gao et al. (1999) reported that induced expression of aquaporins in the primed seeds was involved in the enhancement of seed germination under stress conditions. Based on studies of Arabidopsis seed germination, it was proposed that aquaporins are mainly responsible for the third phase of water uptake (Wiligen et al., 2006). Our results demonstrate that genes encoding for aquaporins, plasma membrane intrinsic proteins (PIP) are involved in canola seed germination. BN10224 (aquaporin PIP2B) and BN10387 (aquaporin PIP1B) genes were only up-regulated in germinated seeds irrespective of the treatments, but not in un-germinated seeds. It appears that these genes act as housekeeping genes and are involved in the third phase of water uptake. In contrast, Bn11437, a third aquaporin (PIP2C) gene

was up-regulated in both germinated and un-germinated seeds imbibed in water and GA; however, it was only up-regulated in germinated seeds imbibed in the saline solution and ABA. Possibly, BN11437, may also be involved in the water uptake of the first two phases; however, it was inhibited by the saline solution or ABA in these phases. A fourth aquaporins gene, BN20259 (PIP1C) was up-regulated in both germinated and un-germinated seeds irrespective of treatments except in ABA treated un-germinated seeds. PIP1C may have a similar role as PIP2C; however, ABA specifically inhibits its up-regulation to function in the water uptake of the first two phases. A fifth aquaporin gene, BN15059 (PIP2A) was only up-regulated in germinated seeds imbibed in either the saline solution or ABA but not in water or GA treated seeds.BN15059 may specifically be involved in overcoming the inhibition imposed under stress conditions. A sixth aquaporin gene, BN17472, similar to plasma membrane aquaporin 2b, was only up-regulated in germinated seeds imbibed in ABA, indicating that this gene was ABA specific. As stated above, ABA has been proposed to inhibit phase III water uptake which was essential for the completion of seed germination (Schopfer and Plachy, 1984; Leubner-Metzger et al., 1995), the differential expression of aquaporin genes in both germinated and un-germinated seeds imbibed either in ABA or the saline solution suggests a much broader role of aquaporins in germination than previously considered.

5.5 Conclusion

There were differences in gene expression pattern between germinated and un-geminated seeds. Gene expression for un-geminated seeds imbibed in water and GA was very similar, in contrast to seeds imbibed in either the saline solution or ABA. Gene expression for germinated seeds was very similar for seed imbibed in either water, GA or

the saline solution compared to seeds imbibed in ABA. While, there were differences in gene expression in germinated seeds due to the treatments, these differences were not as great as compared to their un-germinated counterparts. LEA and stress related genes, hydrolase genes, hormone related genes and some specific seed germination related genes were identified and their expression profiles indicated their roles in the seed germination.

6. General Discussion

Low soil temperatures and salinity are considered to be major factors limiting canola seed germination, emergence, and stand establishment in Western Canada. While information about the control of seed germination has greatly increased, knowledge about the response of hormones and genes involved under abiotic stress conditions is minimal. In order to understand the regulatory roles of hormones and genes on canola seed germination at low temperature, we analyzed the effects of salinity, osmoticum, seed coat (or testa) and exogenous hormones on germination. In addition, we investigated the role of hormones and genes in canola seeds imbibed at 8 ºC by using HPLC-ESI/MS/MS and microarray analysis methods, respectively.

Salinity and reduced water potential dramatically reduced the germination rate and percent of germinable seeds. However, the germination of *Brasicca napus* seeds was more sensitive to PEG solutions than saline solutions at the same osmotic potential. There has been considerable interest in yellow seed canola due to its higher oil, proteins and fibre content compared to the black seed line (Rakow, personal communication; Burbulis et al., 2005). In our studies, we established that the black seed canola line exhibited higher seed vigor at 8 ºC than the yellow seed line although both lines were obtained from the same location and harvested at the same year. The seed coat has been shown to be a major barrier to radicle protrusion for many seeds (Debeaujon et al., 2000; Bewley, 1997b; Leubner-Metzger, 2002; Kucera et al., 2005). In our study, the seed coat restricted seed germination at low temperature and this inhibitory effect was more apparent in the yellow seed line. It has been well established that hormones play important roles in seed germination (Kucera et al., 2005). In our study, GA_{4+7} stimulated

seed germination and ABA inhibited seed germination at low temperature. Ethylene is broadly implicated in promoting seed germination and is antagonistic to ABA (Kepczynski and Kepczynska, 1997). However, when AVG, an ethylene inhibitor was applied, seed germination was not affected, indicating ethylene may not be essential for canola seed germination or AVG was not effective in this study. Numerous studies have demonstrated increased concentrations of salinity induce a proportional increase in ABA in plants (Munns and Sharp, 1993; Cramer and Quarrie, 2002; Sharp and LeNoble, 2002) including *Brassica* species (He and Cramer, 1996). Our result suggests that the inhibitory effect of the saline solution is not due to elevated levels of ABA or perhaps the ABA inhibitor, fluridone was not an effective inhibitor on ABA biosynthesis in canola seed. In our study, fusicoccin (FC) completely overcame the inhibitory effect of ABA, while GA only partially overcame ABA inhibition, suggesting those two compounds may be acting at a similar receptor site, but different from the GA site. The inhibitor GA biosynthesis, PAC, completely inhibited the germination of both lines; however, GA completely overcame this inhibitory effect, FC had only a marginal effect on PAC inhibition. The finding that GA_{4+7} completely overcame PAC inhibition suggests that GA_{4+7} is a major bioactive gibberellin for canola seed germination and its effect is different from FC. The stimulation of germination by GA_{4+7} was greatly reduced in seeds subjected to three weeks of controlled deterioration. Therefore it appears that at the early stages of controlled deterioration, GA is either limiting or its reception sites are impaired.

It is well known that hormones play important roles in seed germination (Karssen et al., 1983, 1989; Nambara et al., 1991; Hilhorst and Karssen, 1992; Debeaujon and Koornneef, 2000; Clerkx et al., 2003; Kucera et al., 2005). Previous studies have shown

that seed germination potential may be regulated by ABA levels, GA levels or the ratio between them (Jacobsen et al., 2002; Ali-Rachedi et al., 2004; Ogawa et al., 2003; Yamauchi et al., 2004). Our physiological studies also support this concept. To further investigate hormonal roles on seed germination, we profiled by HPLC-ESI/MS/MS ABA, ABA metabolites, gibberellins, auxins and cytokinins during germination of canola seeds (cv. N89-53) imbibed at 8 °C in either water, GA_{4+7}, a buffered saline solution or 50 μM ABA in both germinated seeds and un-germinated seeds at different stages of germination. In our study, reduced ABA levels and increased GA_4 contents were observed in seeds that germinated at 8 ºC irrespective of the treatment. This result indicates that these two hormones are involved in canola seed germination at low temperature. Higher ABA levels were detected in un-germinated seeds compared to germinated seeds in all the treatments, indicating there is a threshold level of ABA controlling seed germination. Although ABA level declined in imbibed seeds, the catabolic pathways varied with the treatments. Changes in ABA metabolites as PA, DPA, 7′OH-ABA and ABA-GE revealed that the 8` hydroxylation is the preferred pathway for ABA catabolism in canola seeds imbibed in water at 8 ºC. Higher PA with lower DPA in germinated seeds imbibed in the saline solution indicates that conversion of PA to DPA in seeds is affected by the saline solution. Both ABA-GE and DPA dramatically increased in seeds imbibed in ABA, indicating ABA itself can activate its catabolic pathway. GA_4 was lower in seeds imbibed in the saline solution compared to seeds imbibed in water. This results indicates that the salinity delayed seed germination is partially induced by its inhibitory effect on GA_4 biosynthesis. Our studies also provide evidence that there is interaction between GA and ABA in seed germination. ABA

inhibited GA_4 biosynthesis, whereas, GA had no effect on ABA biosynthesis; however, GA alters the ABA catabolic pathway.

Gene expression patterns via proteomic and transcriptomic analysis aid in the identification of genes involved in germination (Soeda et al., 2005; Nakabayashi et al., 2005; Gallardo et al., 2001, 2002). Profiles of gene expression in canola seeds imbibed at 8 ºC in either water, GA_{4+7}, a saline solution or ABA were identified by microarray analysis. PCA and gene hierarchical clustering revealed that there were significant differences in gene expression pattern between germinated and un-geminated seeds. Gene expression patterns for un-geminated seeds imbibed in water and GA were very similar, in contrast to seeds imbibed in either the saline solution or ABA. Gene expression for geminated seeds was very similar for seed imbibed in either water, GA or the saline solution compared to seeds imbibed in ABA. While, there were differences in gene expression in germinated seeds due to the treatments, these differences were not as great as compared to their un-germinated counterparts. LEA and stress related genes, hydrolase genes, hormone-related genes and some specific seed germination related genes were identified and their expression pattern varied with the treatment. The relationship between gene expression and seed germination stages indicates important roles of these genes in seed germination.

In summary, mechanisms of seed germination and their response to stressful condition were revealed by our physiological, hormonal and transcriptomic studies on canola seed germination. Information from these studies could be used to predict seed germination and may be used by plant breeders to select superior genotype.

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